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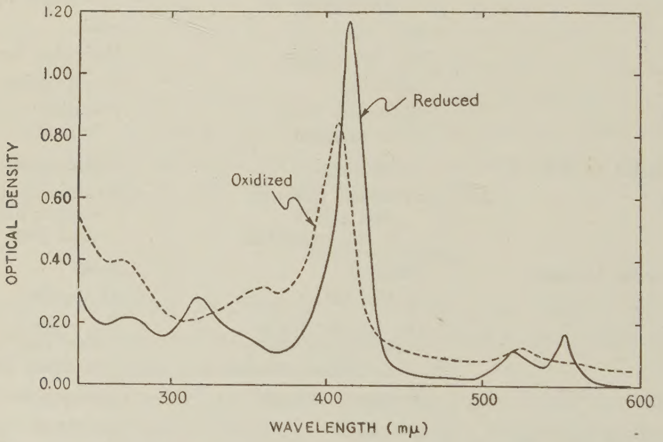

FIG. 1. Absorption spectra of "*Porphyra tenera* cytochrome 553".

The Journal of Biochemistry, Vol. 46, No. 9, 1959

REACTION MECHANISM OF ANIMAL CARBOLIGASE

I. PURIFICATION METHOD AND SUBSTRATES*

By TSUNEO OKUYAMA

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(Received for publication, April 22, 1958)

Carboligase was discovered by C. Neuberg (*1*), but W. Dirscherl denied the presence of the enzyme (*2*, *3*). Thus, D. Green *et al.* (*4*), postulated his particulate preparation to be an animal carboxylase, which had carboligatic activity. Recently, R. S. Schweet, *et al.* (*5*, *7*) and E. Juni, *et al.* (*6*) showed that the pyruvic oxidase could catalyze acetoin formation from pyruvate under anaerobic conditions, while S. Ochoa, *et al.* (*8*) stated that the carboxylase and pyruvic oxidase were different enzymes. The above works chiefly concerned animal enzyme preparations and they are not quite pure in any case. However, I. Gunsalus (*9*) clarified the mechanism of a reaction catalyzed by bacterial pyruvic oxidase; the pyruvic oxidase was a complex of some enzymes, each of which had no pyruvic-oxidase activity.

In this communication, the evidence for the presence of carboligase, which has no pyruvic-oxidase activity, the purification method for the enzyme, and some discussions on the substrates are described.

EXPERIMENTAL

Materials—Cocarboxylase (TPP) (purity: 85 per cent) was purchased from Wako Pure Chemical Industries, Ltd. Sodium pyruvate was prepared by the procedure of Robertson (*10*) from pyruvic acid distilled under a reduced pressure. Sodium glyoxylate was prepared by the procedure of Norman (*21*). Sodium salt of phenyl keto acid was prepared by the procedure of Corson, *et al.* (*22*). Diacetyl was purchased from Kanto Chemicals Co., Ltd. α-Ketoglutaric acid was purchased from Wako Pure Chemical Industries, Ltd. Formaldehyde solution was prepared by dissolving para-formaldehyde. Benzaldehyde was distilled and stored in the sealed tube until use. Acetaldehyde was usually freshly distilled and instantly dissolved in water to a concentration of 0.5 *M*.

Estimation Method—Pyruvate was estimated by the cerium sulfate method by means of Warburg manometer (*11*). Acetoin (acetylmethylcarbinol) was estimated by the method of Eggleton (*12*) after its oxidation to diacetyl with $FeCl_3$. Assay for enzymatic activity was carried out usually with the following system: A substrate mixture of 0.1 ml. of 0.5 *M* sodium pyruvate and 0.1 ml. of 0.5 *M* acetaldehyde in the side arm was tipped into a mixture of 1.0 ml. of enzyme solution, 0.1 ml. of 0.085 per cent cocarboxylase, 0.1 ml. of 0.02 *M* $MnCl_2$ and water to make the total volume of 3.1 ml, after incubation for about 10 minutes at 37.5° in a Warburg reaction vessel. Gas phase was a mixture of 95 per cent N_2 and 5 per cent CO_2. CO_2 evolution was measured for 60 minutes at ten-minute intervals.

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* A part of this paper was presented at the 11th Symposium on Enzyme Chemistry (July, 1955), the Symposium of Vitaminological Society of Japan (Nov. 1954), and at the Annual Meeting of the Chemical Society of Japan (April, 1955).

** Present Adress. Department of Chemistry, Faculty of Science, Tokyo Metropolitan University, Tokyo.

Enzyme Unit—One unit was defined as the amount of the enzyme which was able to catalyze the evolution of 1 μM of CO_2 for 30 minutes under the above-mentioned conditions.

Purification Method for Carboligase—For solubilization of the enzyme, the freezing-thawing method was applied to the Green's suspension which was described in the previous reports (*13*, *23*), but the following method of preparation was found to be more convenient and gave a higher yield. The fresh left ventricle of a pig heart was minced by the previously cooled meat chopper. The mince was thoroughly washed about 5 times with five-folds volumes of ice-cold distilled water. After the washing, it was pressed to remove as much water as possible. This is the pulp of mince, which can be kept for some weeks in a frozen state in dry ice. A mixture of 160 g. of the pulp, 80 ml. of 0.5 *M* phosphate buffer solution (pH 6.0), 80 g. of crushed ice, and 80 ml. of cold distilled water was placed in the goblet of the mixer (Kenwood) and homogenized for two minutes, and further two minutes with 80 g. of additional crushed ice. The treatment was repeated four times (the total of crushed ice used, being 320 g.).

The homogenate was placed in a two-liter flask (egg type) and freezed rapidly in acetone-dry ice mixture (at about −50° or lower). After the frozen material cracked in the flask, it was thawed slowly at 15–20° under tap water. The freezing-thawing treatment, was repeated about 10 times. After this treatment, the homogenate became less viscous and the color became darker than that of the original. It was centrifuged for 30 minutes at 20,000 r.p.m. in a cool state (Spinco Centrifuge L-type). The supernatant was fractionated with ammonium sulfate and the fraction was taken between 0.1 and 0.5 saturation. It was dissolved in 40 ml. of 0.5 *M* phosphate buffer solution (pH 6.0) and the fraction from 0.25 to 0.4 saturation of ammonium sulfate was taken at the second fractionation. After repeating the fractionation with ammonium sulfate, and dissolving the fraction in 0.5 *M* phosphate buffer, a clear, golden yellow solution, which had 5–10 units per ml, was obtained. The yield was 25 per cent of the initial material. The yield of the carbolygase depends on the number of freezing-thawing treatment; the yield from ten treatments was two to three folds of that from five treatments. It easily lost its activity by precipitation at the isoelectric point of pH 4.5 or by heating at 50° for two minutes, but it could be stored in a frozen state in dry ice for about two months. Carboligase is adsorbed on active alumina (Wako Pure Chemicals Industries Ltd.) and the enzyme adsorbed on alumina showed the carboligatic activity in that condition, though it was not so strong. It could not be eluted from alumina.

RESULTS

Absorption Spectrum of Carboligase Preparation—The enzyme preparation, precipitated between 0.2 to 0.5 saturation of ammonium sulfate, has a golden yellow color and its absorption spectrum, which is shown in Fig. 1, has a peak at the wave length of 410 mμ, which corresponds to that of cytochrome c (oxidative form) (*15*). Although there was some possibility that the enzyme preparation was still contaminated with the cytochrome c which might take part in the oxidative reaction of pyruvate, as described by O'kane, *et al.* (*16*) on the bacterial particulate pyruvic oxidase, it seemed impossible, because the amount of contaminated cytochrome c was only small, when it was computed from the optical absorption, and moreover, the change of absorption spectrum in the reaction state was scarcely observed near the wave length of 410 mμ.

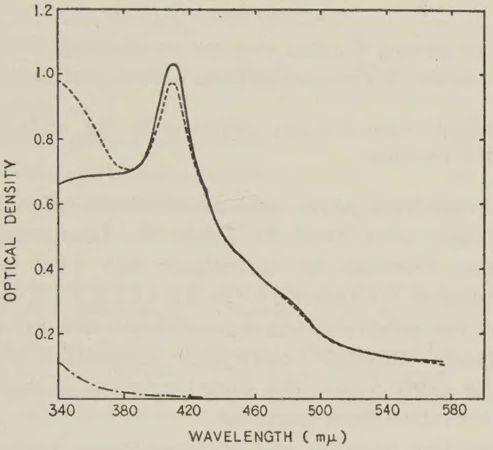

FIG. 1. Absorption spectrum of the concentrated carboligase. Electrospectrophotometer: Beckman Model DU. pH 6.0, temperature, 25°; enzyme, about 30 units/3.5 ml. ——, enzyme solution 3 ml. and distilled water 0.5 ml. ----, enzyme solution 3 ml. and a mixture of substrates and coenzymes (0.085 per cent TPP 0.1 ml., 0.02 M $CoCl_2$ 0.1 ml., 0.5 M pyruvate 0.1 ml. and 0.5 M acetaldehyde 0.1 ml.)

Carboligase without Pyruvic Oxidase Activity—The enzyme preparation has no pyruvic oxidase activity even in the presence of electron acceptors such as methylene blue, 2,6-dichlorophenol-indophenol, molecular oxygen, or potassium ferricyanide. Oxygen uptake was measured in a gas phase of oxygen at pH 6.0 and 7.4, but O_2 uptake was not observed.

In this case, possibility of inactivation of the enzyme by the reaction product, *e.g.* hydrogen peroxide, could be denied, for the CO_2 evolution from pyruvate, which was estimated as a control, was observed regularly. The results of this reaction is listed in Table I. Similar result was observed in the presence of hydrogen carriers such as methylene blue and 2,6-dichlorophenol-indophenol. When ferricyanide was used as an acceptor, the consumption of ferricyanide was estimated by the change in optical density at the wave length of 410 mμ, but in this case, too, the oxidation was not observed.

Dissociation Constant for the Carboligase and TPP—The catalytic acetoin formation by carboligase was usually observed in three cases of substrates, *e.g.*

TABLE I

Balance Sheet of Carboligatic Reaction

Expt No.	Reaction time (*min.*)	Pyruvate consumption (μM)	CO_2 evolution[1] (μM)	Acetoin formation	O_2 uptake (μM)
1	90	6.0	5.3	2.7	0
2	180	11.0	10.1	5.2	0

The amount of enzyme, 6 units; reaction temperature, 37.5°; pH 7.4; gas phase, air; the amount of pyruvate, 100 μM. Other conditions are the same as the usual one.

1) Carbon dioxide evolution was corrected by the addition of 0.5 ml. of 6 N H_2SO_4 after the reaction.

pyruvate alone, acetaldehyde alone, and acetaldehyde plus pyruvate, though the substrate specificity was listed in Table II. Using these three kinds of substrate, dissociation constants for carboligase and TPP were estimated by means of the formulae of Lineweaver-Burk (*17*). The results are shown in Fig. 2. When the substrate was acetaldehyde, the initial velocity of the reaction was measured through colorimetric estimation of the amount of acetoin formed. In other cases, the initial velocity was estimated indirectly through the CO_2 evolution from pyruvate.

Different dissociation constant was obtained for each substrate. It suggests that the combination of TPP with carboligase is also effected by substrates.

From these K_s values in Fig. 2, it was found that the reaction between acetaldehyde and pyruvate also occurs in a mixed substrate.

Relationship between Enzyme Concentration and Reaction Velocity—The initial reaction velocity was measured at various concentrations of the enzyme, and the result is shown in Fig. 3. The apparent affinity of pyruvate to the enzyme is weaker than that in the presence of acetaldehyde at a lower concentration of the enzyme. Accordingly, it appears that there are some different steps in the pathway of the reaction, when the substrate is pyruvate plus acetaldehyde. Thus, there must be a competition between aldehyde and pyrurate. It is also plausible from the next experiment that the binding sites of pyruvate and acetaldehyde are probably the same.

Competition of Aldehyde and Pyruvate—The competition in the early stage of the reaction, was measured through CO_2 evolution from pyruvate, as shown in Fig. 4. Benzaldehyde was used instead of acetaldehyde, because acetaldehyde is volatile and spontaneously mixed with the enzyme mixture in the main compartment before the measuring was started. In curve A, the acceleration of CO_2 evolution was observed for about ten minutes, after which the reaction velocity became constant. It could be explained as follows: All the active sites of the enzyme were occupied at first by pyruvate molecules alone and when benzaldehyde was added as an acceptor of aldehyde, CO_2 evolution was activated extraordinarily, but, when some of the active sites were occupied by

TABLE II

Substrate Specificity of Animal Carboligase

Substrate	CO_2 evolution (*μl/hr*)
α-Ketoglutaric acid	0
α-Ketoglutaric acid+acetaldehyde	0
Phenylketo acid	0
Phenylketo acid+acetaldehyde	0
Glyoxylic acid	0
Glyoxylic acid+acetaldehyde	0
Pyruvic acid	71
Pyruvic acid+acetaldehyde	294
Pyruvic acid+formaldehyde	88
Pyruvic acid+benzaldehyde	257
Pyruvic acid+diacetyl	265

The reaction system was the same as described in "Method", except for the amount of substrates. The amount of keto acid, sodium salt of which was used, was 100 μM for the single substrate, and 50 μM for the mixed substrate. The amounts of acetaldehyde, formaldehyde and diacetyl were 50 μM, respectively. The amount of benzaldehyde was 1 mM, since benzaldehyde was hardly soluble in water. The enzyme units used were 6 units.

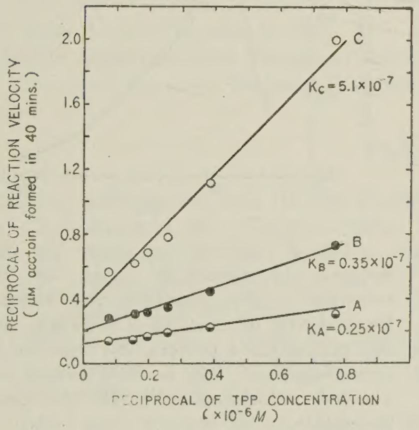

FIG. 2. The effect of substrates on the dissociation constant of carboligase-TPP complex.

A: Substrate; pyruvate 50 μM and acetaldehyde 50 μM.

B: Substrate; acetaldehyde 100 μM.

C: Substrate; pyruvate 100 μM. pH 6.0, 37.5°.

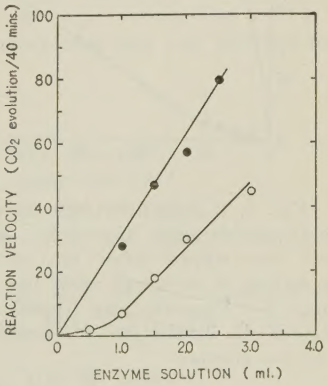

FIG. 3. Relationship between concentration of the enzyme and the reaction velocity. Reaction velocity was estimated by CO_2 evolution by means of Warburg manometer. The reaction system was the same as described in the "Method", except for the amount of enzyme and the total volume of reaction system.

—○— pyruvate plus acetaldehyde,

—●— pyruvate.

benzaldehyde, too, the velocity of CO_2 evolution dropped to the usual level. In curve B, the retardation of CO_2 evolution occurred at first for ten minutes. It could be explained also in the analogous way as above. In the experiment in curve B where aldehyde was added previously, the weak inactivation of carboligase was observed by the action of aldehyde itself to the enzyme protein.

pH-Activity Curve for Acetaldehyde—The optimal pH for acetoin formation from acetaldehyde was at 6.0, as shown in Fig. 5. It is the same value with the optimal pH of the acetoin formation from pyruvate, or from pyruvate plus acetaldehyde for animal carboligase, and comparable with the optimal pH 5.9 for becterial carboxylase (*8*) and with the optimal pH 6.3 for wheat germ carboxylase (*19*). However, it is entirely different from the optimal pH 7.4 for pyruvic oxidase of the pigeon breast muscle (*14*) or of pig heart muscle (*4*).

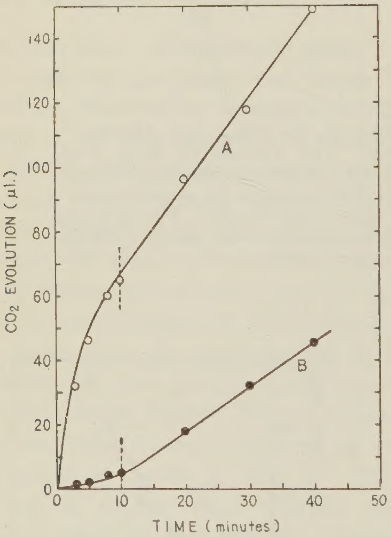

FIG. 4. Competition between pyruvate and benzaldehyde. Curve A: Benzaldehyde was tipped after ten minutes of incubation of pyruvate with the enzyme. Curve B: Pyruvate was tipped after ten minutes of incubation of banzaldehyde with the enzyme.

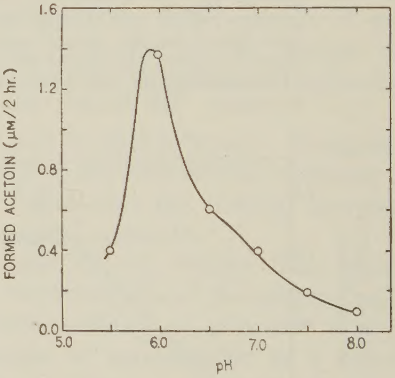

FIG. 5. pH-activity curve for carboligase (acetaldehyde used as the substrate). The reaction was stopped by addition of 1 ml. of 2 *N* H_2SO_4 and the precipitated protein was removed by centrifugation. The acetoin formed was assayed colorimetrically by the usual procedure.

DISCUSSION

The carboligase, which was prepared by the above-described procedure, was free from TPP, but not free from metallic ions. However, repeated salting out with ammonium sulfate, gradually eliminated metallic ions.

This carboligase did not catalyze the oxidation of pyruvate under the conditions described by R. S. Schweet, *et al.* (*5*). They (*7*) announced that their pyruvate oxidase preparation had the molecular weight of about four million, which is somewhat higher value than that of usual enzyme proteins. If the reaction mechanism for bacterial pyruvic oxidase proposed by Gunsalus (*9*) also could be applied in the case of animal tissues, the pyruvic oxidase of animal tissues would be still a complex, and the carboligase described in the present paper may be a component of those pyruvic oxidases. The carboligase has higher specificity toward α-keto acids than toward aldehydes, that is, α-ketoglutaric acid and phenyl keto acid could not be a substrate at all, but formaldehyde, acetaldehyde, propionaldehyde, benzaldehyde, and so on, could be a substrate, though the reactivity was varied. Moreover, aldehyde was more reactive than pyruvate (Fig. 2). Thus the true substrate for carboligase is aldehyde rather than α-keto acid. This fact is an evidence for transcarboligation which is catalyzed by the same enzyme (*20*, *23*).

SUMMARY

1. A simplified purification method for carboligase from pig heart muscle was described.

2. The purified carboligase did not catalyze the oxidation of sodium pyruvate even in the pyresence of any oxidants, *i.e.* under the conditions of Schweet, *et al.* and Juni, *et al.*

3. From the comparison of pyruvate and acetaldehyde, it seems that acetaldehyde rather than pyruvate is the true substrate of carboligase.

The author wishes to express a grateful acknowledgement to Prof. S. Akabori of Osaka University and to Prof. Y. Yamamura of Kyushyu University for their valuable suggestions and discussions throughout the present investigation and to Mr. S. Ikuta of Sasayama National Hospital for his continuous help and kind advices.

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ACTIVATION AND INHIBITION OF ADENOSINETRIPHOSPHATASE ACTIVITY OF MYOSIN B BY PYROPHOSPHATE

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(*From the Department of Physiology, Sapporo Medical College, Sapporo*)

(Received for publication, August 12, 1958)

The combination of myosin B with ATP* has been inferred to be essential for the physical change of myosin B particles. The fact that PP, at lower temperatures, induces a similar change of myosin B (0.6 M KCl solution) has been discovered by Mommaerts (*1*) and investigated in detail by Tonomura *et al.* (*2*). On the other hand, Tonomura *et al.* (*3*) reported in their earlier paper that the ATPase activity of myosin B (0.48 M KCl; pH 6.5), was inhibited by PP in the presence of $CaCl_2$. In seeming contradiction with this finding, Gallop *et al.* (*4*) discovered an activating effect of PP on "crystalline" myosin ATPase. The behavior of PP towards ATPase action of myosin B has been reinvestigated by Uchida *et al.* (*5*) with special reference to its inhibitory effect on the turbidity change of myosin B solution induced by ATP.

The purpose of the present paper is to report the results of our more recent investigations on the effect, activatory as well as inhibitory, of PP on ATPase activity of myosin B.

EXPERIMENTAL

Myosin B was prepared from rabbit muscle by extraction with Weber's solution for 24 hours and purified through three successive precipitation-and-dissolutions with 0.06 M KCl and 0.6 M KCl. "Crystalline" myosin was prepared according to the method of Szent-Györgyi (*6*). Protein concentration was estimated with the micro-Kjeldahl method (factor: 6.25). ATP was a commercial product (Sigma, crystalline disodium salt). PP was recrystallized from the commercial product and was used as 0.1 M solution, the pH was adjusted to 7.0 with 1 N HCl.

The estimation of ATPase activity was carried out in a reaction mixture of the following composition; 0.04 ml. to 0.32 ml. of myosin B solution (5 mg. protein per ml.), 0.8 ml. of Tris-HCl buffer or Tris-maleate buffer containing KCl, varied volume of KCl solution (2 M) to make final KCl-concentrations as indicated in the protocoll, 0.4 ml. of Na_2ATP-solution of varied concentration, 0.2 ml. of $CaCl_2$-solution and 0.2 ml. of PP-solution, and water to make the final volume of 2.0 ml. In the control experiment, water was substituted for the solution of PP and $CaCl_2$. The reaction mixture was preincubated in a constant-temperature bath (±0.1°) for 7 minutes. Then, the ATP-solution was added which had been previously equilibrated to the temperature of the experiment.

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* The following abbreviations will be used; ATP for adenosine triphosphate, ATPase for adenosine triphosphatase and PP for sodium pyrophosphate.

After a measured period of reaction, the reaction was stopped by addition of 2 ml. of 5 per cent trichloroacetic acid. The mixture was filtered and 2 ml of the filtrate was analyzed for orthophosphate by the Fiske-Subbarow method. The color development was carried out at 20°.

Similar experiments were also carried out with "crystalline" myosin.

RESULTS

Effect of Enzyme Concentration and Addition of PP—Fig. 1 shows the rate of ATPase reaction with varied concentrations of myosin B, and in the presence or absence of 10^{-3} *M* PP. In accord with previous findings (*7*, *8*), the rate of ATPase reaction in the initial phase of the reaction was found to be high and liable to variations. After about one minute, a steady level of the reaction was reached. The amount of inorganic phosphate liberated during the initial phase was found to increase with increase in enzyme concentration. However when the enzyme concentration was lowered, the above stated velocity change became usually indistinct. In the conditions studied, no inorganic pyrophosphatase in myosin B preparation was observed.

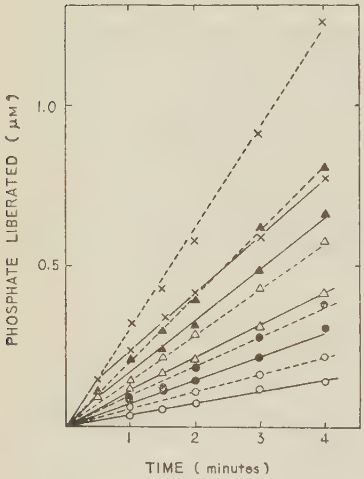

FIG. 1. Rate of ATP hydrolysis with varied myosin B concentrations (25°). Each reaction mixture contains 10^{-3} *M* ATP, 0.6 *M* KCl, 0.08 *M* Tris-HCl buffer (pH 7.2) and enzyme in the total volume of 2 ml. in the presence (broken line) or absence (solid line) of 10^{-3} *M* PP. The enzyme concentration in μg. of protein per ml. is given by ○, 100; ●, 200; △, 300; ▲, 400 ×, 600.

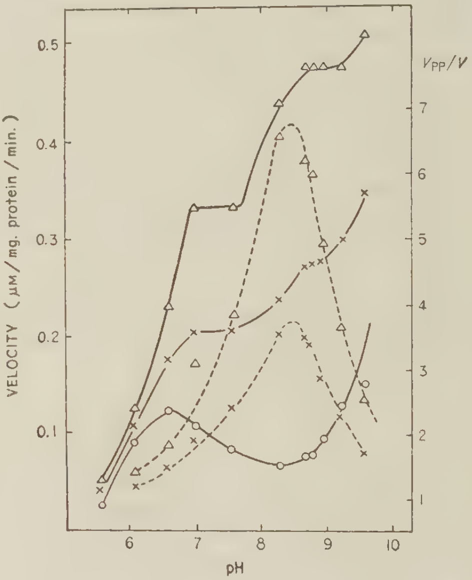

FIG. 2. Effect of PP on the ATPase activity of myosin B as influenced by pH of reaction medium (0.6 *M* KCl, 25°). Reaction mixture contains 300 μg. per ml. of myosin B, 5×10^{-4} *M* ATP, 0.6 *M* KCl and 0.08 *M* Tris-maleate buffer. Signs; ○, control; ×, 10^{-3} *M* PP; △, 5×10^{-3} *M* PP. Broken lines are the ratio of the velocity in the presence of PP to that in the absence of PP.

Fig. 1 shows that PP (10^{-3} M) accelerates the steady rate of ATPase reaction in the total range of myosin B concentration studied. The initial reaction rate was also accelerated by PP in a range of myosin B concentrations less than 300 μg. per ml. The hydrolysis of ATP in the presence of 10^{-3} M PP, assumes a steady rate from the beginning of the reaction. The same was found to be the case, when "crystalline" myosin was used as ATPase. In the following, the velocity of ATP hydrolysis, in the absence of PP, was computed from the tangent of the time course curve for the reaction time greater than two minutes.

Influence of PP on pH-Activity Relationship—In Fig. 2 the pH-activity curves for the ATPase reaction of myosin B are shown as influenced by the presence of PP. It is clearly illustrated in the figure that in the absence of PP, the activity shows an optimum at pH 6.5, a minimum at pH 8.3, and a steep increase towards alkaline side (pH>8.5). The pH-activity curves were essentially the same with various buffering systems (*viz.*, veronal-acetate buffer, Tris-HCl buffer, Tris-maleate buffer and Tris-acetate buffer).

Accelerating effect of PP on the ATPase activity was always noticed over the whole pH-range studied. However, the extent of the activation varied with the pH of the medium. On plotting the ratios of the velocity in the presence, to that in the absence, of PP against pH of the medium, a bell-shaped curve is obtained with an optimum at pH 8.5 (Fig. 2).

Effect of Substrate Concentration and Temperature—The effect of substrate concentration on the reaction rate was also investigated with the purpose of estimating the affinity of the system towards the substrate according to Lineweaver and Burk (*9*). The figure shows that at 25° the addition of 10^{-3} M PP caused a marked decrease in the apparent Michaelis constant, K_m, and no less significant increase in the maximum reaction rate, V_m. On addition of 10^{-3} M $CaCl_2$, the value for the apparent K_m decreased, and V_m increased. When "crystalline" myosin was used as ATPase, both K_m and V_m showed increase on addition of PP (10^{-3} M; more markedly with 5×10^{-3} M).

At 5°, on the other hand, PP acts as an inhibitor towards ATPase action of myosin B. The inhibition was found to be competitive with ATP (Fig. 4). When "crystalline" myosin was used as ATPase, there was also a competitive inhibition of the reaction caused by 10^{-3} M PP. In this case, the apparent K_m increased from 7.3×10^{-5} M to 3.6×10^{-4} M; the value for V_m remained unchanged by the addition of PP.

Similar sets of experiments were run at 20°, 15° and 10°. It was discovered that ATPase activity of myosin B was accelerated by addition of 10^{-3} M PP at temperatures higher than 10°, and inhibited at 5°. The accelerating effect of PP on the ATPase activity of "crystalline" myosin was also found to be limited to temperatures higher than 15°. At 15°, the acceleration by 10^{-3} M PP was obtained also with "crystalline" myosin, when ATP concentration was more than 2×10^{-4} M, but the same concentration of PP caused an inhibition at concentrations of ATP lower than 2×10^{-4} M.

The logarithms of the reciprocal of the apparent Michaelis constant,

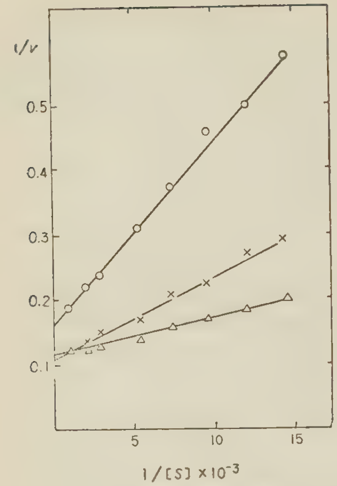

FIG. 3. Effect of substrate concentration on the velocity of ATP hydrolysis at 25°. Myosin B concentration is 300μg. per ml. Other conditions are the same as those given in Fig. 1. Signs; ○, control; △, 10^{-3} M CaCl$_2$; ×, 10 M PP.

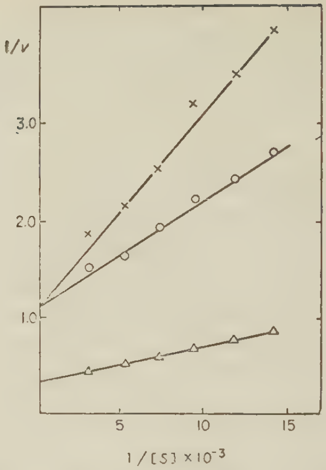

FIG. 4. Effect of substrate concentration at 5°. Experimental conditions are the same as those given in Eig. 3. Signs; ○, control; △, 10^{-3} M CaCl$_2$; ×, 10^{-3} M PP.

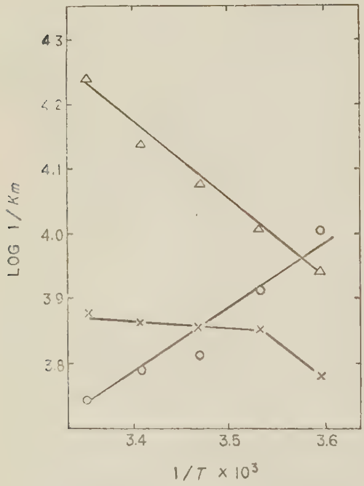

FIG. 5. Log $1/K_m$ as a function of $1/T$. Signs; ○, control; △, 10^{-3} M CaCl$_2$; ×, 10^{-3} M PP.

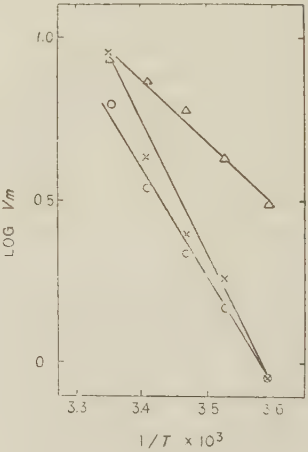

FIG. 6. Log V_m as a function of $1/T$. Signs; ○, control; △, 10^{-3} M CaCl$_2$; ×, 10^{-3} M PP.

K_m, and the maximum reaction rate, V_m, are plotted against $1/T$, respectively, in Figs. 5 and 6. The values of K_m in the absence of PP and CaCl$_2$ increased with temperature, while the values in the presence of 10^{-3} M CaCl$_2$ decreased with increase in temperature, each set of data giving a linear log K_m-$1/T$

plot from which the values for enthalpy change were computed, $\Delta H=-4.6$ kcal and $\Delta H_{CaCl_2}=5.6$ kcal. The same values in the presence of 10^{-3} *M* PP remained almost unchanged at temperatures higher than 10°, but shows a decrease towards 5° at which temperature PP exhibited an inhibitory effect on the ATPase activity of myosin B. In all the cases studied, *Vm* increased with rise in temperature each giving a linear Arrhenius plot, from which the activation energy of the reaction was computed to be as follows, $E=15$ kcal, $E_{CaCl_2}=9$ kcal and $E_{pp}=17.5$ kcal. These values obtained with myosin B in the presence of $CaCl_2$ are smaller than those reported by Ouellet *et al.* (*10*).

Effect of PP in the Presence of NaCl—The effects of KCl and NaCl on the myosin B-ATPase were investigated in the presence of PP (Table I). When NaCl was substituted for KCl, the grade of activation caused by addition of PP decreased. Furthermore, the ATPase activity of myosin B, not only in the presence, but also in the absence of PP was found to be lowered by increasing NaCl concentration. The results are similar to those of Bowen (*11*). On the other hand, the viscosity change of myosin B solution* in 0.6 *M* NaCl as caused by 2×10^{-4} *M* ATP was lowered by 10^{-2} *M* PP addition to 60 per cent that of the control (25°).

TABLE I

Effect of NaCl and KCl on ATPase Activity of Myosin B in the Presence of PP

The values are in μ moles of phosphate split per mg. of myosin B per min. pH 7.0 (Tris-maleate buffer), 25°, 5×10^{-4} *M* ATP.

Concentration of PP	0	10^{-3} *M*	5×10^{-3} *M*
KCl 0.6 *M*	0.108	0.205	0.318
KCl 0.3 *M*+NaCl 0.3 *M*	0.043	0.046	0.056
NaCl 0.6 *M*	0.007	0.007	0.008

DISCUSSION

The above described experimental data indicate that the mode of action of PP on myosin B and "crystalline" myosin with respect to the ATPase activity of these proteins, is probably different at higher and lower temperatures each other. At 25°, PP activated the ATPase activity of myosin B, suggesting that PP and ATP combine separately at different sites of myosin B molecule. At 20° and 23°, on the other hand, it has been previously observed that PP inhibited competitively the viscosity response and the fall in light-scattering of myosin B solution as caused by ATP (*5*). If this fact is to be interpreted to mean that both PP and ATP combine with a common site of myosin B molecule, the site of myosin B molecule responsible for its ATPase action must be different from the site at which ATP combines with the protein to induce the physical changes of the myosin B molecule.

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* The viscosity was measured at pH 7.1 (0.08 *M* Tris-HCl buffer) using a viscosimeter of Ostwald type, containing 5 ml. of myosin B solution (2.0 mg. protein per ml.).

It was known that, at 5°, PP by itself induced the physical change of myosin B particles (*1*, *2*). As described above, at 5°, it inhibited competitively the ATPase activity.

Thus, there is a possibility that PP combines in several ways with myosin B under the different temperature conditions: the combination may take place at the same site where ATP combines with the enzyme: or at some different site causing the change of the affinity of the myosin B molecule towards ATP.

On the other hand, the combination of ATP with myosin B may take place in two ways; i) ATP may combine at a site of myosin B molecule to be hydrolyzed (ATPase-site), or, ii) ATP may combine at another site of myosin B molecule to induce, a physical change characteristic to myosin B.

Bowen and Kerwin (*11*) have reported on the influence of pH on EDTA effect, and Morales *et al.* (*12*) have presented the pH-V_m curve of myosin B treated with S-β-aminoethylisothiouronium in the presence of Ca^{++}. Both pH curves of Bowen and of Morales seem to be in accord with the results in the present study. Nihei *et al.* (*13*) have reported that the ratios of the velocity of ATP hydrolysis by myosin B treated with S-β-aminoethylisothiouronium in the presence, to that in the absence, of Ca^{++} recalculated from the data of Morales *et al.* will also give a bell-shaped curve when plotted against pH. It will be justified to assume here that there are same features in common with the mode of action of PP, EDTA and S-β-aminoethylisothiouronium.

The effect of monovalent cations may be due to the hydration of the ion as has been discussed by Kachmar and Boyer (*14*) in the case of pyruvate phosphoferase. Further work is required to give a decision concerning this point.

SUMMARY

The effects of PP on the ATPase activity of myosin B and "crystalline" myosin were studied.

1. At concentrations of myosin B higher than 300 μg. per ml., it was observed that the rate of phosphate liberation from ATP in the initial phase of the reaction was greater than that in the later, steady phase of the reaction. In the presence of PP, the hydrolysis of ATP by myosin B keeps a steady rate from the beginning of the reaction.

2. The effect of PP on the pH-activity relationship of ATPase reaction of myosin B was investigated at 25°. PP accelerates the ATPase reaction of myosin B in all the pH ranges studied. The degree of the acceleration by PP was found to be a function of pH, having a maximum at pH 8.3 under the experimental conditions studied.

3. The steady rate of the ATPase reaction in concentrations of ATP ranging from 7×10^{-5} M to 10^{-3} M was estimated in the presence or absence

of PP and $CaCl_2$. The ATPases of myosin B and "crystalline" myosin were found to be accelerated by 10^{-3} M PP at temperatures higher than 10° and 15°, respectively: at 5° there was an inhibition caused by PP (10^{-3} M) which was found to be competitive with respect to the concentration of substrate, ATP.

4. The logarithms of V_m and $1/K_m$ give linear plots when plotted against $1/T$. In the presence of PP, the plot of reciprocal of apparent K_m against $1/T$ shows a break at a critical temperature reflecting different heats of the formation of enzyme-substrate complex above and below this temperature.

5. The effect of NaCl on the ATPase activity of myosin B in the presence or absence of PP was investigated. The degree of acceleration of ATPase by PP was found to decrease with increasing concentration of NaCl.

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ON THE TRANSPHOSPHORYLATION REACTIONS CATALYZED BY GLUCOSE-1-PHOSPHATE PHOSPHOTRANSFERASE OF ESCHERICHIA COLI

I. ENZYMATIC PHOSPHORYLATION OF RIBOFLAVIN

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(Received for publication, October 7, 1958)

Recently, the authors have isolated from dried cells of *Escherichia coli*, an enzyme preparation which requires G-1-P* as specific phosphate donor and catalyzes transfer of the phosphate group to various nucleosides, including B_2. In its nature the enzyme resembles "nucleoside phosphotransferase", previously designated by Chargaff (*1*), however the enzyme preparation obtained by the authors has a higher specificity towards G-1-P, and the name "G-1-P phosphotransferase" is proposed for this enzyme. This enzyme system would actually be responsible for the biosynthesis of nucleotides, especially FMN, during bacterial growth.

In the present paper the properties of this phosphotransferase, and the conditions for enzymatic formation of FMN through the transphosphorylation will be described.

Another enzymatic phosphorylation of B_2 has been studied by Kearney (*2*) using flavokinase prepared from yeast and also by the present authors with the cell-free extract of *E. coli* (3), and the following mechanism has been postulated;

$$B_2 + \text{ATP} \longrightarrow \text{FMN} + \text{ADP}.$$

The phosphorylating mechanism of B_2 by G-1-P phosphotransferase as described in this paper, should be regarded as the "general" pathway, primarily proposed by Chargaff (*1*), of transphosphorylation between nucleosides and G-1-P.

EXPERIMENTAL

Materials—β G-1-P was prepared according to Reithel (*4*), araboflavin (6,7-dimethyl-9-(D-1′-arabityl) isoalloxazine), 6-methyl-9-(D-1′-ribityl)-isoalloxazine and hydroxyethylisoalloxazine were the kind gifts of Prof. Takata. Isoriboflavin (5,6-dimethyl-9-(D-1′-ribityl) isoalloxazine) was generously supplied by the Tokyo-Tanabe

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* The following abbreviations are used in this paper: B_2, riboflavin: FMN, flavin mononucleotide. G-1-P, glucose-1-phosphate: ATP, adenosine triphosphate; ADP, adenosine diphosphate; G-6-P, glucose-6-phosphate; FDP, fructose-1,6-diphosphate.

Co., Ltd. B_2-ketoic acid (1,2-dihydro-6,7-dimethyl-2-keto-1-D-ribityl-3-quinoxaline carboxylic acid) was synthesized by Surrey's method (*5*). The other compounds were commercial products.

Organism—The organism used in this study was *E. coli neapolitanus* (B-25). The bacteria were grown with agitation at 30° in a medium containing 1.5 per cent bouillon, 1.5 per cent maltose and 0.5 per cent yeast extract (pH 7.2) for 15 hours. The cells were harvested by Sharples centrifuge, washed with distilled water, and dried with an electric fan. The dried cells were kept in a desiccator at 0°.

Methods—Inorganic orthophosphate was determined by the method of Fiske-Subbarow (*6*), and glucose by Somogyi-Nelson's method (*7*). The microdetermination of FMN was carried out by the method of Kearney (*2*), and the phosphorylation of the B_2 analogues was estimated principally by the same procedure as employed in previous experiments on the transglycosidation reaction (*8*) after separation by paper chromatography using a solvent system of butanol-pyridine-water (6 : 4 : 3). The corresponding nucleotide was extracted with distilled water and was determined by measuring the optical density at 450 mμ, employing the Beckman DU spectrophotometer.

RESULTS

Purification of G-1-P Phosphotransferase—The following procedures were always carried out at 0°. One g. of the dried cells was ground in a porcelain mortar with 4 g. of pyrex glass powder and 5 ml. of 0.2 *N* acetate buffer (pH 5.4). The homogeneous paste was diluted to 45 ml. with the acetate buffer and centrifuged for 30 minutes at 6,000 r.p.m.

The opalescent supernatant (35 ml.) was treated with solid ammonium sulfate to give 0.30 saturation. After 15 minutes, the precipitate was removed off by centrifugation, and the ammonium sulfate concentration was raised to 0.80 saturation. After centrifugation, the precipitate was dissolved in 20 ml. of the acetate buffer.

The fractionation with ammonium sulfate was repeated twice in the same manner as described above.

To the buffered solution (pH 5.4) *N* acetic acid was added with vigorous stirring to make a pH of 4.5. The precipitate was centrifuged off, and the supernatant fluid was immediately adjusted to pH 5.4 by careful addition of *N* KOH solution and dialyzed against a 0.2 *N* acetate buffer (pH 5.4) for 18 hours. When a precipitate appeared, it was removed by centrifugation.

The dialyzed solution (20 ml.) was adjusted to pH 4.5 with *N* acetic acid and treated with 5 g. of Fuller's earth. After stirring for 1 hour, the suspension was centrifuged, the precipitate was washed with distilled water and the enzyme was eluted by stirring with 10 ml. of 0.1 *N* citrate buffer (pH 8.6) for about 3 hours. The eluate was kept in a refrigerator.

The activity of the purified enzyme was increased to about 30 times that of the crude extract, when prepared according to the procedure described above. The preparation retained its activity for a week when it was stored at 0°.

Table I shows the summarized results of a typical fractionation and the successive steps of purification. The enzyme unit was defined as the amount of the enzyme which transferred 1 μM phosphate from G-1-P to B_2 in 1 hour under the conditions given in the Table.

Since the enzyme has not yet been prepared in a pure state, the preparation reveals also some phosphatase activity which rapidly hydrolyzes the donor substrate, G-1-P. Further investigations as to whether transphosphorylating and hydrolytic activities would be possibly separated or not, will be dealt with in the following papers of this series.

TABLE I

Summary of Fractionation of Enzyme

Fraction	(*ml.*)	Protein (*mg./ml.*)	Enzyme (*unit/ml.*)	Enzyme (*unit/mg. protein*)
Crude extract	35	18.2	4.15	0.23
Ammonium sulfate fractionation	20	14.8	7.00	0.47
Acid precipitation and dialysis	20	5.2	6.20	1.20
Fuller's earth adsorption and elution	10	1.2	8.67	7.22

The enzyme was assayed in a 2.5 ml. reaction mixture, containing 2×10^{-4} *M* B_2, 0.01 *M* G-1-P, 0.1 *M* acetate buffer (pH 5.4) and 1 ml. of the enzyme. The reaction mixture was incubated for 1 hour at 30°.

TABLE II

Donor Specificity of G-1-P Phosphotransferase

Donor substrate	Phosphate liberated (mM)	FMN synthesized (μM)
α G-1-P	4.76	13.2
β G-1-P	2.75	6.1
FDP	0.32	0
G-6-P	0.38	0
α Glycerophosphate	0.01	0
β Glycerophosphate	0.01	0
p-Nitrophenylphosphate	0.14	0
ATP	0.02	0
Pyrophosphate	0	0
5′-Adenylate	0	0

The purified enzyme was used. The reaction conditions were as described in Table I, and substrates were used in each case at the final concentration of 1×10^{-2} *M*.

Donor Specificity—The most interesting properties of the enzyme was the absolute specificity towards the donor substrate. As shown in Table II, the enzyme preparation has the ability to utilize only G-1-P as phosphate donor, and it has been observed that the other phosphomonoesters such as G-6-P, FDP and phenylphosphate, play no role as donor. Moreover, ATP, pyrophosphate and adenylate are not utilized by this enzyme as phosphate donor.

Furthermore, from the fact that the activity of β-type of G-1-P was only half that of α-type, stereochemical specificity of the enzyme to glucosidic linkage of the donor would possibly be indicated.

Effect of pH—The activity/pH curves for both transphosphorylase- and phosphatase-activities of the enzyme preparation showed a marked similarity (Fig. 1), the pH optimum being found to be in the range of 5.2 to 5.4. As the phosphorylation ratio, that is the molar ratio of the phosphate transferred to the glucose liberated, was markedly decreased on the alkaline side, it was suggested that the relative activity of phosphatase would be higher than transphosphorylase in this range of pH.

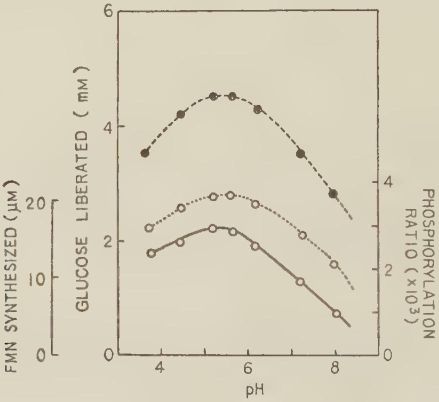

FIG. 1. Effect of pH on the activity of G-1-P phosphotransferase.

The reaction mixture (2.5 ml.) containing G-1-P B_2 and the purified enzyme in acetate buffer as described in Table I, was incubated at 30° for 1 hour.

--●-- glucose liberated, —○— FMN synthesized, --○-- phosphorylation ratio.

Effect of the Chemical Reagents—Among the experiments with several chemical reagents which were regarded to affect the transphosphorylation, some typical results are summarized in Table III.

The magnesium ion and other alkaline earth ions did not appreciably affect the enzyme activity. Sodium fluoride proved to be a powerful inhibitor of the enzyme action, although the degree of inhibition depends on the substrate concentration and pH of the reaction system. Molybdate of 0.5 per cent concentration also inhibited the enzyme action to a degree of about 100 per cent.

Inorganic phosphate revealed inhibition to the transphosphorylation as well as to the hydrolysis of G-1-P.

TABLE III

Effect of Mg, NaF and the Other Inhibitors on G-1-P Phosphotransferase Activity

The reaction mixture (2.5 ml.) containing G-1-P, B_2 and the purified enzyme in acetate buffer as described in Table I, was incubated at 30° for 1 hour.

Addition	Concentration (10^{-3} M)	Inhibition (%) of	
		Glucose liberation	FMN synthesis
None	—	0	0
$MgSO_4$	0.1	11	7
	0.5	6	
$CaCl_2$	0.5	14	
EDTA	2	2	3
NaF	25	100	100
$(NH_4)_2MoO_4$	(0.5%)	100	100
	(0.1%)	47	
KH_2AsO_4	5	18	25
KH_2PO_4	5	9	13
	10	41	
	50	100	100
Glucose	200	51	84
Fructose	200	12	24
Maltose	200	4	20

The addition of glucose produced a striking effect on this reaction system. The remarkable inhibition by glucose, especially to the transphosphorylating activity, seems to be competitive to the donor, G-1-P. This fact, together with the above mentioned result of donor specificity, suggests that glucose moiety in the G-1-P molecule would be responsible for the enzyme action. Fructose, maltose and sugar alcohols had hardly any effect under the same conditions.

Further investigations with SH reagents, oxidants and heavy metals will be described in the following paper.

Effect of the Donor and the Acceptor Concentration—Increasing the donor concentration resulted in a higher yield of phosphorylated product, hardly changing the phosphorylation ratio as shown in Fig. 2. The system seemed to be almost saturated with G-1-P at the concentration of about 1×10^{-2} M.

Fig. 3 illustrates the increase in phosphorylation ratio linear with the acceptor concentration.

Phosphorylation of B_2 Analogues—As shown in Table IV, the enzyme reacted similarly on the B_2 analogues as on B_2 itself, except for B_2-ketoic acid and hydroxyethylisoalloxazine which was the only monohydroxyl compound among the tested analogues.

Though there is a report that yeast flavokinase failed to phosphorylate isoriboflavin (*9*), the reactivity of the G-1-P phosphotransferase presented here to isoriboflavin suggests that this transferring system would possibly be available to the phosphorylation of various natural substances such as nucleosides.

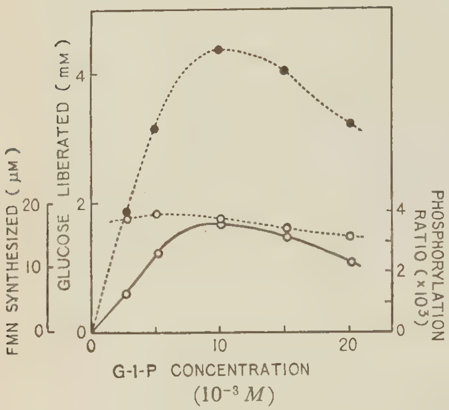

FIG. 2. Effect of donor concentration on FMN synthesis by G-1-P phosphotransferase. The reaction mixture (2.5 ml.) containing B_2, the purified enzyme and G-1-P of various concentrations, in acetate buffer (pH 5.4) as described in Table I, was incubated at 30° for 1 hour. --●-- glucose liberated, —○— FMN synthesized, --○-- phosphorylation ratio.

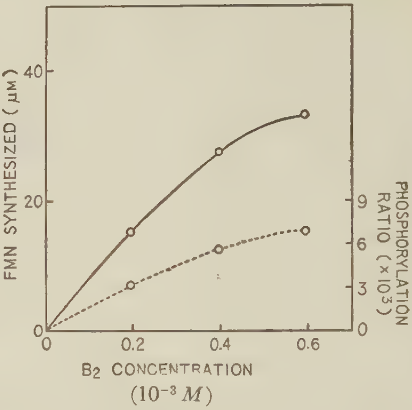

FIG. 3. Effect of B_2 concentration on FMN synthesis by G-1-P phosphotransferase. The reaction mixture (2.5 ml.) containing G-1-P, the purified enzyme and B_2 of various concentrations, in acetate buffer (pH 5.4) as described in Table I, was incubated at 30° for 1 hour. —○— FMN synthesized, --○-- phosphorylation ratio.

TABLE IV

Phosphorylation of Various Analogues of B_2 By G-1-P Phosphotransferase

The reaction mixture (2.5 ml.) containing G-1-P or either of analogue of B_2 and the purified enzyme in acetate buffer as described in Table I, was incubated at 30° for 1 hour.

Analogue of B_2	Phosphorylated B_2 analogue snythesized (μM)	Percentage of transphosphorylation
Riboflavin	12.2	100
Araboflavin	11.4	93
6-Methyl-9-(D-1′-ribityl)-isoalloxazine	11.0	93
Isoriboflavin	8.9	73
Hydroxyethylisoalloxazine	0	0
B_2-ketoic acid	0	0

DISCUSSION

The results mentioned above show the presence of transphosphorylase which reacts specifically with G-1-P and transfers the phosphate group to B_2.

Phosphate transfer reactions which can proceed in the absence of a high energy donor, were discovered by Axelrod (*10*), who demonstrated that phenylphosphate served as a phosphate donor to methanol and other alcohols in certain phosphatase-catalyzed reactions. Meyerhof and Green (*11*) and Morton (*12*) extended these observations to show that a variety of substances including ATP and phosphopyruvate, as well as phenylphosphate, can serve as phosphate donor, while a number of different alcohols and sugars could be acceptors, and that various phosphatases from different sources were active in these reactions.

It became clear that instead of the hydrolysis of the donor, transfer takes place, and that for this to occur high concentrations of the acceptor are necessary. The fact that transfer occurs is a clear indication of the preferential attack of the donor by the alcoholic hydroxyl group of the acceptor rather than the hydroxyl anion.

However it is open to question whether these phosphatase-catalyzed transfer reactions may play any physiological role *in vivo* phosphate transfer because of their nonspecificity with regard to donor as well as acceptor and of the requirement for high concentrations of acceptor in order that the reaction may proceed to a measurable extent. The reaction system of the G-1-P phosphotransferase utilizes low energy phosphate, namely G-1-P, as phosphate donor, and proceeds with rather low concentrations of acceptor. Moreover, the phosphorylations of B_2 and its analogues proceed at similar ratios as in the case of the flavokinase reported by Kearney (*2*). The authors assume, therefore, that the G-1-P phosphotransferase system differs by the specific ability from the above mentioned phosphate transfer.

Chargaff (*13*) reported recently an interesting finding that the phosphate transferring activity was separated from phosphatase, and demonstrated with this transferase preparation the specific formation of nucleotides. These workers indicated also that the most efficient donor was phenylphosphate which does not exist in the organism.

The G-1-P phosphotransferase preparation involves also some G-1-P phosphatase activity at the present stage of purification, but both activities would be possibly separated by ion exchange chromatography, as will be reported in the following paper.

The acceptor specificity of this enzyme is now being investigated by the authors, and it has been clarified that natural nucleosides play the role of efficient acceptor in the reaction system of the G-1-P phosphotransferase. These results will also be reported in the following papers.

On the other hand, Doudoroff (*14*) has shown that G-1-P is involved as the intermediate in the metabolic process of maltose by *E. coli.* It seems that the bacterium, especially that growing in the presence of maltose, utilizes G-1-P to synthesize several phosphate compounds which are essential for bacterial growth. Certainly, G-1-P phosphotransferase activity has been found to be enriched in the bacterial cells when grown in the medium containing maltose.

SUMMARY

1. An enzyme preparation which catalyzes the specific transphosphorylation from G-1-P to certain acceptors such as B_2 was isolated from the dried cells of *E. coli.*

2. Experimental results demonstrate that the phosphorylation proceeds effectively even at very low concentrations of the substrates.

3. The enzyme activity was not affected by the addition of magnesium ion, but inhibited by fluoride, phosphate and glucose. Inhibitory effect of glucose is attributable to the competitive action to G-1-P.

4. Various analogues of B_2 were also phosphorylated in this system under the same conditions as those used for the phosphorylation of B_2.

5. The authors suggest the significant role of the G-1-P phosphotransferase in the biosynthesis of the phosphate compounds which are essential for bacterial growth.

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DISTRIBUTION OF I^{131} INSULIN INJECTED INTO PORTAL VEIN OF RAT*

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(Received for publication, October 10, 1958)

It is of great importance to investigate the site of action insulin in mammalian body. In recent studies with eviscerated dog (*1*, *2*) and isolated diaphragma (*3*, *4*) it has been revealed that insulin accelerates the penetration of sugar into muscular tissue. However, these experiments can hardly be regarded as physiological in that the control of liver is excluded, for under physiological conditions insulin which is produced in the pancreatic islet cells enters into blood stream of portal vein and is transported to the liver. If it is true in the intact mammalian body that the hypoglycemic action of insulin is to accelerate the penetration of sugar into cells of extrahepatic tissues, the insulin would be expected to distribute to all the parts of body after passing through the liver. The present study was undertaken in order to investigate the distribution of insulin in the animal body after it was administered into portal vein of rats by using insulin preparation labeled with I^{131}.

EXPERIMENTAL

Insulin Labeled with I^{131}—A commercial sample of insulin was purified paper-chromatographically by the method of A. Light and M. Simpson (*5*), but no attempt was made to remove the contaminating glucogen. The purified insulin was labeled with I^{131} according to Stadie *et al.* (*6*).

The iodinated samples were dialyzed against running water for two to three days and the portion which was precipitated at pH 5.0 and soluble at pH 3.0 was collected. This preparation was proved to retain the hormonal activity when tested by the use of rabbit, but was less stable. Most of its activity was lost by standing for two days at 4°. The preparation contained about 0.1 to 0.4 mole of iodine per mole of insulin and a trichloracetic acid soluble fraction which amounted to a few per cent of the total radioactivity.

I^{131}-cytochrome c was prepared from highly purified cytochrome c of horse heart muscle.

I^{131}-human serum albumin was prepared in the same way as used in the iodination of insulin.

I^{131}-activity was measured by means of well type scintillation counter (Scientific

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* The major part of this work was reported at the Endocrinological Society of Japan at Tokyo, in February 1958.

Research Institute Ltd., Tokyo, Japan).

The abdominal cavity of the rats which had been fed *ad libitum* was opened, and the labeled preparations in a volume of 0.2 to 1.0 ml. were injected into mesenterial vein directed to portal vein. Injected samples were neutralized to pH 7.0 with dilute NaOH just before to use. The rats were sacrificed 8 to 10 minutes after the injection and I^{131} activity of various organs was measured.

RESULTS

Table I shows the distribution of the radioactivity of I^{131}-insulin injected into portal vein. About 90 per cent of the activity was recovered in the liver

TABLE I

Distribution of I^{131}-Insulin Injected into Portal Vein

Tissue	Rat No.	Weight (*g.*)	Radio-activity (*c.p.m.*)	Specific activity (*c.p.m./g.*)	Incorporation (%)
Liver	1	5.2	21490	4134	90.0
	7	4.5	8000	177	94.0
	8	5.0	12870	2574	89.0
Kidney	1	1.0	148	148	0.6
	8	1.1	128	116	0.8
Diaphragm	1	0.6	60	100	0.2
	8	—	—	—	—
Heart	1	0.6	56	92	0.2
	8	0.5	35	70	0.2
Lung	1	2.1	512	243	2.1
	8	0.9	150	166	
Adrenal	1	0.05	22	440	0.09
	8	—	—	—	—
Spleen	1	1.30	104	80	0.4
	8	—	—	—	—
Thigh	1	—	—	43	—
Muscle (chest)	1	—	—	78	—
Blood	1	—	—	300	—
	8	—	—	243	—
Others	1	100.0	1300	130	5.5
	7	110.0	50	4	6.0
	8	116.0	1141	98	7.9

Insulin was injected into portal vein of rats; No. 1, about 900 μg. of insulin, No. 7 about 2 μg. of insulin, and No. 8, 50 μg. After 8 to 10 minuts, the animals were decapitated and eviscerated, then the radio-activity was estimated.

TABLE II

Distribution of I^{131}-Cytochrome c, I^{131}-Human Albumin and Denaturated I^{131}-Human Albumin Injected into Portal Vein

Tissue	Rat No.	Weight (*g.*)	Radioactivity (*c.p.m.*)	Specific activity (*c.p.m./g.*)	Incorporation (%)
Liver	3	4.5	536	119	7.5
	4	4.0	2085	521	29.7
	5	7.0	1769	252	17.0
	6	5.5	1657	301	15.00
Kindly	3	0.8	164	205	2.3
	4	0.8	275	343	3.9
	5	1.2	252	210	2.5
	6	1.0	180	180	1.6
Heart	3	0.7	215	307	3.0
	4	0.5	317	634	4.5
	5	0.5	139	248	1.3
	6	0.5	95	190	0.8
Pulmonum	3	0.7	236	332	3.3
	4	0.8	328	410	7.6
	5	0.7	141	201	1.4
	6		—	—	—
Spleen	3	—	—	—	—
	4	—	—	—	—
	5	0.5	22	44	0.2
	6	0.5	100	200	0.9
Pancreas	3	—	—	—	—
	4	—	—	—	—
	5	0.6	122	203	1.2
	6	—	—	—	—
Blood	3	—	—	941	—
	4	—	—	—	—
	5	—	—	340	—
	6	—	—	315	—
Others	3	100.0	5461	54	76.9
	4	115.0	3992	34	57.0
	5	120.0	7912	82	79.0
	6	110.0	8826	79	80.0

No. 3, about 90 μg. of I^{131}-human albumin, No. 5, and No. 6, about 50 μg. of I^{131}-cytochrome c, and No. 4, denatured insoluble I^{131}-human albumin as suspension were injected.

and the activity could not be washed out by perfusion of the liver with a physiological saline.

As control experiments, I^{131}-cytochrome c and I^{131}-human albumin were injected. In these cases the radioactivity of the injected preparations was distributed uniformly throughout the whole body as shown in Table II. In contrast to the undenatured active I^{131}-insulin, the labeled hormone which had lost their biological activity by allowing to stand in a refrigerator was

Table III

Distribution of Denatured I^{131}-Insulin Injected into Portal Vein

Tissue	Rat No.	Weight (*g.*)	Radio-activity (*c.p.m.*)	Specific activity (*c.p.m./g.*)	Percent incorporation (%)
Liver	2	14.0	4019	287	18.9
	9	5.0	3200	640	32.6
Kidney	2	2.8	139	41	0.6
	9	1.0	210	210	2.1
Heart	2	2.5	137	54	0.6
	9	0.5	161	322	1.6
Pulmonum	2	1.6	387	129	1.8
	9	0.6	228	380	2.3
Spleen	2	0.5	136	272	0.6
	9	—	—	—	—
Pancreas	2	1.5	413	276	1.9
	9	—	—	—	—
Muscle	2	—	—	109	—
	9	—	—	50	—
Blood	2	—	—	158	—
	9	—	—	480	—
Others	2	181.0	15979	88	75.3
	9	120.0	6071	56	61.9

Denatured I^{131}-insulin in amounts of about 50 μg. calculated as protein was injected into portal vein.

distributed in the same pattern as the control experiments with I^{131}-cytochrome and I^{131}-albumin. No charecteristic accumulation in liver was observed (Table III).

DISCUSSION

Recently, N. D. Lee (*7*) observed that the major part of insulin-I^{131} intravenously administered to rats was recovered in the kidney and the liver. The insulin-I^{131} in the liver was found in the microsomal and the mitochondrial fractions. When alkali inactivated insulin-I^{131} was administered, the incorporation of I^{131} into the microsomal fraction was remarkably decreased, while the incorporation into the mitochondrial fraction showed no decrease. So Lee has supposed the microsomal fraction to be a site of insulin action.

From the results reported in this paper and the Lee's observation, liver seems to be the target organ of insulin in mammalian body.

SUMMARY

Most part of the radioactivity of I^{131}-insulin when injected into portal vein of rats was recovered from liver, while the radioactivity of inactivated I^{131}-insulin, I^{131}-cytochrome c and I^{131}-human albumin all of which had been administered through portal vein was distributed indiscriminately in the bodies. Thus the hepatotropic nature of insulin may be considered probable.

I^{131}-cytochrome c was supplied from Dr. S. Minakami of this Department, Dr. K. Titani, and Dr. H. Ishikura of the Department of Chemistry of the University. Human albumin was supplied from Dr. T. Higashi of the Department of Biochemistry of the University. The authors wish to express their thanks for their kindness.

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STUDIES ON METABOLISM OF AMIDES IN MYCOBACTERIACEAE

II. ENZYMATIC TRANSFER OF NICOTINYL GROUP OF NICOTINAMIDE TO HYDROXYLAMINE IN MYCOBACTERIUM AVIUM

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(Received for publication, November 27, 1958)

Asparto- glutamotransferases are known to catalyze the exchange of the β-aspartyl group of asparagine and the γ-glutamyl group of glutamine with hydroxylamine respectively. They have been found in bacteria (*1–4*), mammalian tissues (*5*), and green plants (*6–8*). For the full activity of these transferases of mammalian tissues and green plants Mn^{++} and phosphate ion are required, whereas bacterial transferase does not require any co-factor, except that of *Proteus vulgaris* which requires Mn^{++} ion.

Recently, Grossowicz and Halpern (*4*) found that the extracts of *Mycobacterium phlei* possess glutamotransferase activity but not aspartotransferase activity.

This report describes experiments on the enzymatic transfer of the nicotinyl group of nicotinamide to hydroxylamine and also deals with the partial purification of the enzyme. The enzyme under consideration will be tentatively called nicotinotransferase in this report.

METHODS AND MATERIALS

Quantitative Analysis—The quantity of hydroxamate was electrophotometrically measured by Lipmann-Tuttle's method (*9*). Protein concentration was measured by Lowry's method (*10*).

Assay of Enzyme Activity—Four ml. of a standard assay mixture contained 200 μM of phosphate or Tris buffer, 50 μM of nicotinamide, 200 μM of hydroxylamine, and the enzyme. The reaction was started by the addition of the enzyme, and the incubation was carried out for 60 minutes at 38°. The blank control, which contained all components except the substrate, produced negligibly small amount of hydroxamate.

Materials Used—The nicotinamide (m.p. 129–130°), benzamide (m.p. 127–129°), L-asparagine, L-glutamine and acetamide were obtained from commercial sources. The acetamide (m.p. 76.5–81.5°) was once recrystalized.

Purification of the Enzyme—*Mycobacterium avium* (strain Takeo) was grown in glycerol-bouillon medium for 5 days. The collected cells were washed and drained as much

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The following abbreviations will be used: ATP, adenosine triphosphate; EDTA, ethylendiaminetetraacetic acid; Tris, tris(hydroxymethyl) aminomethane.

as possible and poured into 10 volumes of acetone previously cooled to −40° by the addition of dry-ice. After brief stirring, the cells were allowed to settle, the supernatant fluid was decanted, and the cells were filtered through cotton-cloth. The filter cake of cells was desiccated in vacuo until the solvent was completely evaporated. In this way, the cells were made into a powder.

Five grams of the acetone-dried cells were finely ground and powdered with a large amount of sea sand, and subsequently extracted with 200 ml. of 1.0 per cent sodium bicarbonate. After aging overnight in a refrigerator, the cells were centrifuged off at 10,000 r.p.m. for 20 minutes at 0°. The supernatant fluid was used as crude extract. Two ml. of 1 per cent protamine sulfate solution were added to 100 ml. of the crude extract. After stirring and standing for 20 minutes, the precipitate formed was centrifuged off at 10,000 r.p.m. for 20 minutes. The supernatant fluid was fractionated with solid ammonium sulfate to 35 per cent saturation under cooling. The precipitate formed was centrifuged off, and solid ammonium sulfate was added to the supernatant fluid to 65 per cent saturation. Again, the precipitate formed was centrifuged and dissolved in a small amount of phosphate buffer (pH. 6.8), dialysed overnight against running water, and then dialysed for 3 hours against 0.001 *M* EDTA solution. Through this operation, the specific activity of the enzyme was raised about 4-fold. (Table I)

TABLE I

Summary of Purification of Nicotinotransferase from Mycobacterium avium

Fraction	Total volume (*ml.*)	Total protein (*mg.*)	Total units* (*unit*)	Specific activity (μM/*hr./mg. protein*)	Yield (%)
Crude extract	300	735	1257	1.71	100
Protamine supernatant	306	638	1350	1.96	107
Ammonium sulfate (35%)	28	64	60	0.94	4.7
Ammonium sulfate (35–65%)	43	82	535	6.52	43.8

Phosphate buffer (pH 6.8), 200 μM; nicotinamide, 50 μM; NH_2OH, 200 μM; total volume, 4.0 ml; 38°, 60 minutes.

* One unit of enzyme activity was defined as the amount of the enzyme which gave 1.0 μM of nicotinohydroxamate for one hour at 38°.

RESULTS

Formation of Hydroxamate as a Function of Time—The results of the experiment for nicotinohydroxamate formation from nicotinamide and hydroxylamine by nicotinotransferase as a function of time showed that the equilibrium was attained in about 90 minutes as shown in Fig. 1.

Effect of Enzyme Quantity on Hydroxamate Formation—Figure 2 shows the effect of various quantities of the enzyme on hydroxamate formation. Less than 1.0 mg. of the enzyme protein gave a linear relationship.

Effect of Hydroxylamine Concentration on Hydroxamate Formation—Fig. 3 gives the relation between hydroxylamine concentration and the amount of hydroxamate formed. Hydroxylamine is required in excess of the equivalent amount of nicotinamide. Michaelis constant for hydroxylamine is tentatively

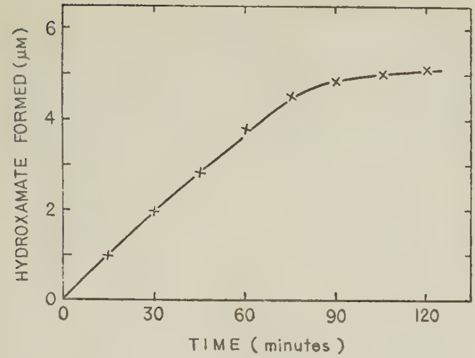

FIG. 1. Rate of hydroxamate formation as a function of time in *Mycobacterium avium*.

Enzyme (ammonium sulfate 65 per cent fraction), 382 μg. of protein; Tris buffer (pH 8.0), 200 μM; nicotinamide, 100 μM; NH_2OH, 200 μM; total volume, 4.0 ml.; 38°.

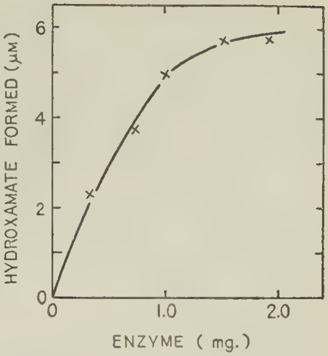

FIG. 2. Effect of enzyme concentration on hydroxamate formation.

Enzyme, (ammonium sulfate 65 per cent fraction), as indicated; Tris buffer (pH 8.0), 200 μM; nicotinamide, 50 μM; NH_2OH, 200 μM; total volume, 4.0 ml.; 38°, 60 minutes.

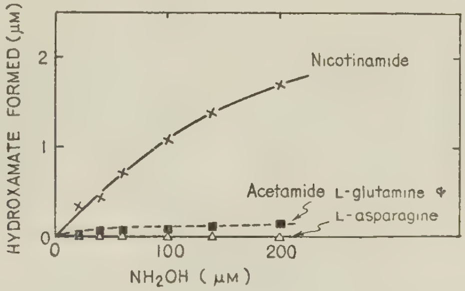

FIG. 3. Effect of hydroxylamine concentration on transferase activity.

Enzyme (ammonium sulfate 65 per cent fraction), 0.1 ml.; phosphate buffer (pH 6.8), 200 μM; nicotinamide, 50 μM; L-asparagine, L-glutamine, 20 μM; acetamide, 500 μM; NH_2OH, as indicated; total volume, 4.0 ml.; 38°, 60 minutes.

calculated: $K_m = 3.0 \times 10^{-2}\ M$.

In this range of hydroxylamine concentration, L-asparagine, and L-glutamine were completely inactive, and acetamide was slightly active for hydroxamate formation.

Effect of Nicotinamide Concentration on Hydroxamate Formation—Fig. 4 shows the effect of various concentrations of nicotinamide on hydroxamate formation by nicotinotransferase. From the data obtained, Michaelis constant for nicotinamide can be tentatively calculated: $K_m = 4.3 \times 10^{-3}\ M$.

Effect of pH on Hydroxamate Formation—The optimum pH of nicotinotransferase was determined with the crude extract and the purified enzyme. The crude extract has the optimum pH at 7.6–7.8, and the purified enzyme at 8.0 (Fig. 5). While glutamotransferase in animal tissue is activated by phosphate ion, nicotinotransferase from *M. avium* requires no phosphate ion, as seen from the lower activity in phosphate buffer than in Tris buffer. Glutamotransferase discovered in *M. phlei* by Grossowicz *et al.* (*4*) is activated by phosphate or arsenate, but nicotinotransferase in *M. avium* is different in this respect.

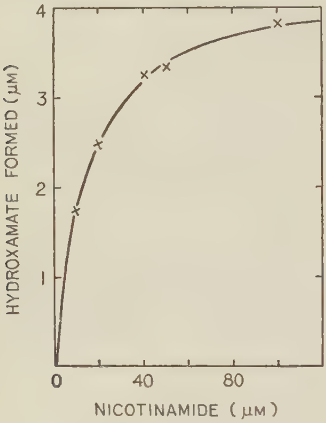

FIG. 4. Effect of nicotinamide concentration on transferase activity.

Enzyme (ammonium sulfate 65 per cent fraction), 382 μg. of protein; Tris buffer (pH 8.0), 200 μM; nicotinamide, as indicated; NH_2-OH, 200 μM; total volume, 4.0 ml.; 38°, 60 minutes.

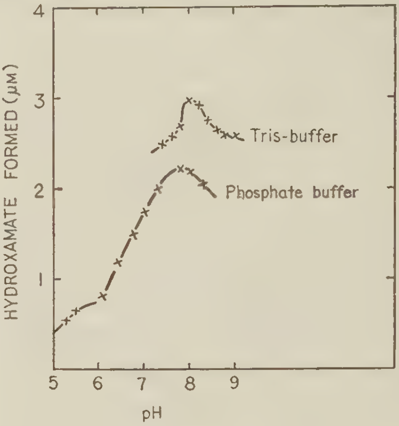

FIG. 5. Effect of pH on nicotinotransferase activity.

Enzyme (ammonium sulfate 65 per cent fraction), 382 μg. of protein phosphate or tris buffer, 200 μM; nicotinamide, 50 μM; NH_2OH, 200 μM; total volume, 4.0 ml.; 38°, 60 minutes.

TABLE II

Effect of Metal Ions on Transferase Activity

Metal ions	Hydroxamate formed (μM)	Per cent
None	1.50	100
Hg^{++}, 1.3×10^{-4} *M*	0.19	13
Cu^{++}, ,,	0.05	3
Zn^{++}, ,,	1.66	111
Cr^{+++}, 2.5×10^{-4} *M*	1.54	103

Enzyme (ammonium sulfate 100 per cent fraction), 223 μg. of protein*; phosphate buffer (pH 6.8), 200 μM; NH_2OH, 200 μM; nicotinamide, 50 μM; total volume, 4.0 ml.; 38°, 60 minutes.

* This value indicates the amount of enzyme which was used for the assay.

Effect of Metal Ions on Hydroxamate Formation—The effect of metal ions was examined with purified enzyme; Table II shows that Hg^{++}, Cu^{++} remarkably inhibited, Ba^{++}, Mn^{++}, Cr^{+++} had no effect, and Ni^{++}, Co^{++}, Ca^{++}, Mg^{++}, Zn^{++}, activated about 10 per cent. Glutamotransferases from plant and animal tissue require Mn^{++} ion, and glutamotransferases from *M. phlei* (*4*) and *Proteus vulgaris* (*11*) also require Mn^{++} ion. However, the absolute requirement for metal ion was not observed with nicotinotransferase from *M. avium* in this stage of purification. This fact corresponds well to non-inhibition by metal catchers.

Effect of Inhibitors on Hydroxamate Formation—The effect of metal catchers was investigated with the purified enzyme. α,α'-Dipyridyl, *o*-phenanthroline, ethylenediaminetetraacetate, and nitroso-R did not show any appreciable inhibition. (Table III)

TABLE III

Effect of Inhibitors on Transferase Activity

Inhibitor (*M*)		Hydroxamate formed (μM)	Per cent to original activity
None		1.77	100
α,α'-Dipyridyl,	5×10^{-5}	1.71	97
o-Phenanthroline	1.3×10^{-4}	1.67	94
EDTA	1.3×10^{-3}	1.57	89
Nitroso-R	2.5×10^{-4}	1.50	85
KCN	2.5×10^{-3}	1.67	94
$NaHSO_3$	1.3×10^{-3}	1.63	92

Enzyme (ammonium sulfate 100 per cent fraction), 223 μg, of protein; NH_2OH, 200 μM; nicotinamide, 50 μM; phosphate buffer (pH 6.8), 200 μM; total volume, 4.0 ml.; 38°, 60 minutes.

TABLE IV

Heat Inactivation of Nicotinotransferase

Treatment	Hydroxamate formed (μM)	Per cent to original activity
None	3.60	100
50°, 5 minutes	0.39	10.8
50°, 10 minutes	0.21	5.8
50°, 15 minutes	0.09	2.5
50°, 20 minutes	0.06	1.6

Enzyme (ammonium sulfate 65 per cent fraction), 382 μg. protein; Tris buffer (pH 8.0) 200 μM; nicotinamide, 50 μM; NH_2OH, 200 μM; total volume, 4.0 ml.; 38°, 60 minutes.

Heat Stability—The purified enzyme was tested for heat stability. Denaturation was observed by treatment at 50° for 5, 10, 15, and 20 minutes. If the enzyme preparation had been stored in a frozen state, it was stable-though it showed slight lowering of activity. (Table IV)

Substrate Specificity—Enzyme activity was measured in each stage of purification with nicotinamide, benzamide, L-asparagine, L-glutamine, and acetamide as substrates. Table V shows that the crude extract from *M. avium* is active on nicotinamide, benzamide, and acetamide and that the activities on benzamide and acetamide are about half as much on nicotinamide. It has not yet been able to conclude whether the individual enzymes exist for each substrate or the same enzyme is active on all of these amides. Transferase for L-asparagine and L-glutamine was found neither in the crude extract nor in the purified fractions.

TABLE V

Hydroxamate Formation from Nicotinamide, L-Asparagine, L-Glutamine, Acetamide and Benzamide

Substrate Fraction	Nicotioamide (50 μM)	L-Asparagine (50 μM)	L-Glutamine (50 μM)	Acetamide (500 μM)	Benzamide (25 μM)
Crude extract 1.23 mg*.	2.10 (μM)	0.06(μM)	0.00(μM)	1.02(μM)	0.99(μM)
Protamine supernatant 1.13 mg*.	2.22	0.00	0.00	0.90	0.78
Ammonium sulfate (35%) 0.51 mg*.	0.48	0.00	0.00	0.45	0.27
Ammonium sulfate (65%) 0.38 mg*.	2.49	0.12	0.00	1.29	1.05
Heated enzyme	0.00	0.00	0.00	0.00	—
Without enzyme	0.00	0.00	0.00	0.00	—

Enzyme as idicated; phosphate (pH 6.8), 200 μM; NH_2OH, 200 μM; total volume, 4.0 ml.; 38°, 60 minutes.

* The values show the amount of protein used.

DISCUSSION

From the results obtained it is evident that nicotinohydroxamate is enzymatically formed from nicotinamide and hydroxylamine. For this enzyme the name nicotinotransferase may be suggested.

In crude extracts from *M. avium*, no hydroxamate was formed from nicotinic acid (50 μM) and hydroxylamine (20, 40, 60, 80, 100, 140, 1200 μM) at pH 5.2 ~ 8.3. No hydroxamate was formed at all from nicotinic acid and hydroxylamine with crude extract containing coenzyme A in the presence of ATP. Also L-aspartic or L-glutamic acid did not form corresponding hydroxamate in the presence of CoA and ATP. Grossowicz *et al.* (*4*) have also reported that there are no asparto- and glutamosynthetase in *M. phlei.*

Kimura (*12*) purified the nicotinamidase extensively from *M. avium*, which was completely inhibited by Hg^{++}, Cu^{++}, Zn^{++}, Cr^{+++}, and Fe^{+++}. The optimum pH of nicotinamidase is 8.3. Therefore, nicotinotransferase is quite different from nicotinamidase in the respect to the inhibition by heavy metal ions and pH optimum.

In *M. phlei* Grossowicz *et al.* (*4*) found L-asparaginase and glutamotransferase but not aspartotransferase and L-glutaminase. It was found also in our laboratory that in *M. phlei* nicotinamidase, L-asparaginase and glutamotransferase exist, while nicotinotransferase, aspartotransferase, and L-glutaminase are absent. It will be shown in another paper that the simultaneous existence of amidase and transferase is not always found in *Mycobacteriaceae*. Finally it could be concluded that in *M. avium* nicotinotransferase is different from nicotinamidase. Also, nicotinotransferase differs from the nicotinohydroxamate hydrolase, as will be shown in another paper now in preparation.

SUMMARY

1. In extracts of *Mycobacterium avium* (strain Takeo), a transferase which transfers nicotinyl group of nicotinamide to hydroxylamine has been found. The enzyme may be named nicotinotransferase. The crude extracts are also active on benzamide and acetamide but half as much active as on nicotinamide. No activity was found on L-asparagine and L-glutamine.

With this extract there occurred no synthesis of hydroxamate from nicotinic acid and hydroxylamine in the presence of ATP.

2. The enzyme was purified about 4-fold and some of its properties are described.

3. It is suggested that the transfer of nicotinamide and the hydrolysis of nicotinamide and nicotinohydroxamate may be catalyzed by different enzymes.

The author is indebted to Prof. T. Sasakawa for his interest and encouragement and to Mr. S. Satoh for his technical assistant during the course of this study.

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THE EFFECT OF DETERGENTS ON OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA

II. THE STIMULATORY EFFECT OF QUATAMIN 24 P ON PHOSPHORYLATION COUPLED WITH THE OXIDATION OF SUCCINATE

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It was previously reported (*1*) that cationic detergents such as Quatamin 24 P greatly stimulated phosphorylation coupled with oxidation of succinate in rat liver mitochondria. This occurred at a low concentration of the detergent which did not cause a change of transparence of the suspension. The present paper gives results of further studies on the stimulatory effect of Quantamin 24 P.

Materials and methods used are as described in the previous report (*1*).

RESULTS

It was shown in Table V of the previous report (*1*) that the increase of phosphorylation coupled with oxidation of succinate was dependent on the ratio of Quatamin 24 P to the concentration of mitochondria. A maximal stimulation of oxidative phosphorylation with succinate as substrate was observed at a relative concentration (*1*) of nearly 1.0, at which the decrease in the optical density of the mitochondrial suspension started to occur. Table I shows the effect of the ratio of Quatamin 24 P to the concentration of mitochondria on oxidative phosphorylation. The stimulatory effect of the detergent on oxidative phosphorylation decreased as the relative concentration decreased. The loss of oxidative phosphorylation was occurred at a relative concentration of more than 1.0. These results suggest that Quatamin 24 P combines stoichiometrically with receptor sites in the mitochondria. Loss of oxidative phosphorylation with succinate at high relative concentration is due to inhibition by a high concentration of the detergent, since addition of 7×10^{-3} per cent (final concentration) of the detergent (which corresponded to 3.5×10^{-2} per cent initial concentration or a relative concentration of 3.5) strongly inhibited oxidative phosphorylation (Table II).

A study was made of the influence of the osmotic pressure of the mitochondrial medium on the effect of Quantamin 24 p. Table III shows

TABLE I

Effect of the Ratio of Quatamin 24 P to Mitochondria on Oxidative Phosphorylation with Succinate as Substrate

Mitochondrial suspension (*ml.*)	Relative concentration	Transparence	Initial concentration of Quantamin 24 P (%)					
			0			7×10^{-3}		
			ΔO[1] (*μ atoms*)	ΔP[2] (*μ atoms*)	P/O	ΔO (*μ atoms*)	ΔP (*μ atoms*)	P/O
0.010	3.5	⧺	1.07	1.11	1.04	0.61	0.33	0.54
0.030	1.2	+	2.88	4.24	1.47	2.44	3.61	1.48
0.050	0.7	±	3.22	5.60	1.74	3.89	6.52	1.68
0.075	0.5	—	4.81	7.64	1.59	5.16	8.30	1.61
0.100	0.4	—	5.26	8.40	1.59	5.55	8.60	1.55

1.0 ml. of mitochondrial suspension is equivalent to 6.0 g. of liver. Experimental conditions are described in the previous paper (*1*).

1) ΔO: Oxygen consumption expressed in *μ atoms*.

2) ΔP: Inorganic phosphate incorporated into organic compounds (*μ atoms*).

TABLE II

Effect of Addition of Quatamin 24 P to the Reaction Medium on Oxidative Phosphorylation of Succinate

Concentration of Quatamin 24 P[1] (%)	ΔO (*μ atoms*)	ΔP (*μ atoms*)	P/O
0	3.10	3.92	1.26
1.4×10^{-3}	3.15	4.00	1.27
2.1×10^{-3}	3.25	4.03	1.24
7.0×10^{-3}	0.94	0.09	—

1) Final concentration in the reaction medium. Experimental conditions are described in the previous paper (*1*).

that stimulation of phosphorylation coupled with oxidation of succinate by Quantamin 24 P is greater in a hypotonic than a hypertonic medium. At an initial concentration of sucrose of 0.075 *M* (hypotonic), addition of Quatamin 24 P gave 42 per cent stimulation and at an initial concentration of 0.225 *M* gave 27 per cent stimulation. Witter and Mink (*2*) reported that synthetic detergents have more effect on the swelling of mitochondria in an alkaline medium in which the mitochondria are inclining to be unstable (*3*, *4*). Therefore a study was made of the influence of pH of the reaction medium on the effect of Quatamin 24 P on oxidative phosphorylation. Fig. 1 shows that Quatamin 24 P stimulates phosphorylation coupled with oxidation of succinate of mitochondria most at pH 7.5~8.0. The above data show that Quatamin 24 P is most effective in increasing oxidative phosphorylation under conditions in which mitochondria are inclining to be unstable. Therefore it is reasonable to assume that the stimulatory effect is due to distortion of the mitochondrial structure by the detergent.

TABLE III

Effect of Tonicity of Mitochondrial Suspension on the Action of Quatamin 24 P

Initial concentration of sucrose[1] (*mole*)	Initial concentration of Quatamin 24 P (%)						Increase in phosphorylation[2] (%)
	0			7.0×10^{-3}			
	ΔO (*μ atoms*)	ΔP (*μ atoms*)	P/O	ΔO (*μ atoms*)	ΔP (*μ atoms*)	P/O	
0.225	4.50	5.71	1.27	5.40	7.26	1.34	27
0.150	4.37	5.94	1.36	5.60	7.50	1.34	26
0.125	3.34	5.56	1.66	4.80	7.42	1.55	33
0.100	3.52	5.40	1.53	5.76	7.34	1.27	38
0.075	4.67	4.74	1.01	5.74	6.73	1.17	42

1) Concentration of sucrose in side arm chamber of Warburg vessel during preincubation.

2) Increase of the percentage of the value of phosphorylation in the mitochondria treated with the detergent to that of intact mitochondria.

Experimental conditions are described in the previous paper (*1*).

Quatamin 24 P may stimulate oxidative phosphorylation in the following ways: A) Some enzyme involved in oxidative phosphorylation system is activated by the detergent. Approach of the detergent to the enzyme is facilitated distortion of mitochondria by the detergent. B) The enzyme system of oxidative phosphorylation which may normally be masked by some factor unmasked by distortion of mitochondrial structure caused by the detergent and can therefore react with the substrate more easily. C) Decrease in activity of oxidative phosphorylation by aging during preincubation at 24° is prevented by the detergent, and, under conditions in which mitochondria are inclining to be unstable, the protecting effect of the detergent seems to stimulate the increase of oxidative phosphorylation. If the stimulatory effect of Quatamin 24 P is due to reason (A), it might be expected that the increase in oxidative phosphorylation would be accompanied by an increase in efficiency of oxidative phosphorylation as judged by the P:O ratio. However, as shown in Tables I, III and Fig. 1, the increase in oxidative phosphorylation of mitochondria treated with the detergent was not accompanied by an increase in the P:O ratio, and occasionally this was slightly decreased.

Though hypotonic treatment of mitochondria promoted the stimulatory effect of Quatamin 24 P on oxidative phosphorylation, thermal treatment of mitochondria did not. Instead it resulted in inhibition by the detergent of phosphorylation coupled with oxidation of succinate, as shown in Table IV. These inhibitory effects of Quatamin 24 P were observed on coupled phosphorylation but not on oxidation of succinate.

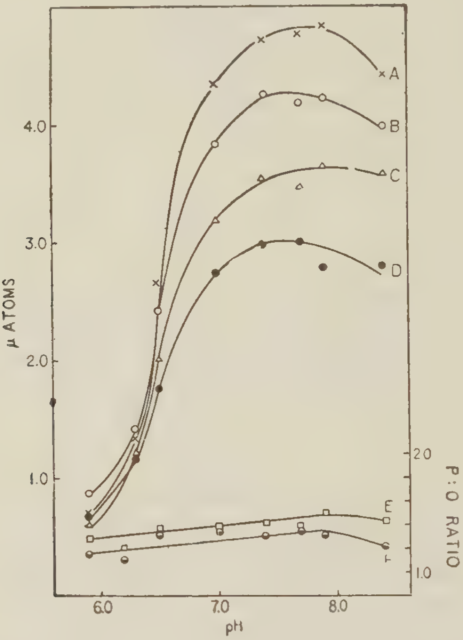

FIG. 1. Effect of pH of the reaction medium on the action of Quatamin 24 P.

The pH of the medium was adjusted with a glass electrode pH meter. The conditions are as in Table III with the exception that 0.02 *M* phosphate buffer, 0.01 *M* ATP and 0.15 *M* sucrose were used for suspension medium.

Curve A, C and F represent respectively, coupled phosphorylation, oxidation and P : O ratio of the mitochondria treated with Quatamin 24 P (initial concentration 7.0×10^{-3} per cent).

Curve B, D and E represent respectively these values for the intact mitochondria.

Tables I and II show that high concentration of Quatamin 24 P strongly inhibited both oxidation of succinate and coupled phosphorylation. However the uncoupling of oxidative phosphorylation caused by a low concentration of the detergent show that the detergent was more inhibitory for coupled phosphorylation than for oxidation. From these results, it is difficult to conceive

TABLE IV

Effect of Aging of Mitochondria on the Action of Quatamin 24 P

Pretreatment at		Initial concentration of Quatamin 24 P (%)					
		0			7.0×10⁻³		
0° (*min.*)	24° (*min.*)	ΔO (*μ atoms*)	ΔP (*μ atoms*)	P/O	ΔO (*μ atoms*)	ΔP (*μ atoms*)	P/O
0	0	4.24	6.77	1.60	4.94	7.80	1.58
50	0	4.30	6.70	1.56	4.75	6.50	1.37
25	25	4.36	6.46	1.48	4.61	6.36	1.38
15	35	4.61	6.26	1.34	5.35	4.06	0.76
0	50	4.90	0.84	0.17	4.42	1.09	0.25

Preincubation of intact mitochondria was carried out first at 0° and then at 24° in 0.3 *M* sucrose. Experimental conditions are described in the previous paper (*1*).

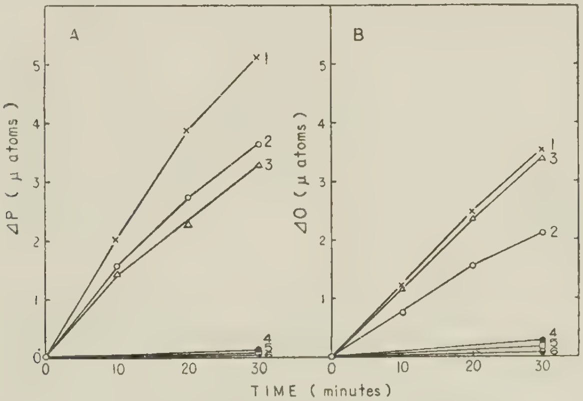

FIG. 2. Time course of reaction in the presence of Quatamin 24 P. The experiment was carried out under the standard conditions described in the previous paper (*1*) with the exception that a concentration of mitochondria equivalent to 2 g. of liver per ml. was used.

Curve 1, 3 and 2 of A represent respectively coupled phosphorylation of mitochondria treated with an initial concentration 7.0×10^{-3} per cent and 1.0×10^{-2} per cent of Quatamin 24 P, and of intact mitochondria.

Curve 5, 6 and 4 represent respectively the endogeneous phosphorylation under the same conditions.

Curves in Fig. B represent the oxidation of succinate under the above conditions.

that some enzyme of the oxidative phosphorylation system is activated by

Quatamin 24 P.

Table IV shows that in intact mitochondria phosphorylation coupled with oxidation of succinate hardly decreases during 25 minutes preincubation at 24°. However, it is interesting that the effect of Quatamin 24 P on intact mitochondria differs from that on mitochondria which have been preincubated for 25 minutes at 24°. Fig. 2 gives the time course for the oxidative phosphorylation of reaction of mitochondria treated Quatamin 24 P and indicates that stimulation occurs in the initial stage of the reaction and the reaction velocity of intact mitochondria hardly decreased during the reaction. Therefore reason (C) by which stimulation of Quatamin 24 P on oxidative phosphorylation might occur, is impossible.

Quatamin 24 P did not prevent the decrease in phosphorylation coupled with oxidation of succinate, but rather increased the instability of the mitochondria. Table V indicates that Quatamin 24 P inhibits rather than stimulates oxidative phosphorylation when the mitochondria are preincubated with the detergent. The result suggests that the mitochondria are distorted by Quatamin 24 P and thus the oxidative phosphorylation system is more labile and easily uncoupled. Therefore, it is concluded that Quatamin 24 P causes distortion of the mitochondria which at first stimulates oxidative phosphorylation, but that since the distorted state of the mitochondria tends to labilize the system, preincubation with detergent results in final inhibition of the system.

TABLE V

Effect of Preincubation of Mitochondria treated with Quatamin 24 P on the Action of the Detergent

Preincubation with Quatamin 24 P (*min.*)	Initial concentration of Quatamin 24 P (%)					
	0			7.0×10^{-3}		
	ΔO (*μ atoms*)	ΔP (*μ atoms*)	P/O	ΔO (*μ atoms*)	ΔP (*μ atoms*)	P/O
5	3.72	5.60	1.50	4.29	6.41	1.50
20	3.56	5.53	1.55	4.24	6.13	1.45
30	3.39	5.11	1.51	4.36	5.06	1.16
45	2.95	4.35	1.47	4.21	0.68	0.16

The mitochondria were preincubated with the detergent in 0.3 *M* sucrose in the side arm chamber of Warburg vessel at 24°.

Experimental conditions are described in the previous paper (*1*).

DISCUSSION

From the above results the following hypothesis is put forward. The activity of the oxidative phosphorylation system is normally masked by some factor. This is removed when the mitochondria are distorted by Quatamin 24 P. The resulting increase in phosphorylation coupled with oxidation of succinate appears only when there is insignificant inhibition by the detergent. Since there is stimulation of both oxidation of succinate and coupled phosphorylation, increase in oxidative phosphorylation is not always accompanied an increase in the P : O ratio. Quatamin 24 P accelerates decrease in oxidative phosphorylation of aged mitochondria, since in these the system has a tendency to disintegrate. The distorted state of the mitochondria causes lability. Therefore, decrease in oxidative phosphorylation is also accelerated by preincubation of the mitochondria with the detergent.

If the reason for stimulation of phosphorylation coupled with oxidation of succinate by Quatamin 24 P is as described above, it may be expected that the detergent would stimulate oxidative phosphorylation of other substrates, such as ascorbate and β-hydroxybutylate. However, Quatamin 24 P did not stimulate phosphorylation with these substrates. Inhibition of oxidative phosphorylation with ascorbate by the detergent may be due to inhibition of coupling of the ascorbate-cytochrome c system with phosphorylation, as discussed previously (*1*). However, it is difficult to explain why Quatamin 24 P does not stimulate phosphorylation coupled with oxidation of β-hydroxybutylate-DPN. Oxidation of β-hydroxybutylate-DPN and coupled phosphorylation were easily inhibited by many detergents (*1*). Cationic detergents such as Quatamin 24 P do not inhibit phosphorylation coupled with oxidation of β-hydroxybutylate-DPN (*1*) as much as other detergents. However, no stimulation by Quatamin 24 P may be connected with a specific phosphorylation step of β-hydroxybutylate-DPN system which is easily inhibited by the detergent.

The stimulatory effect of the detergent on phosphorylation coupled with oxidation of succinate was also observed with cholate but not with digitonin as previously described (*1*).

SUMMARY

1. Quatamin 24 P, a cationic detergent, at low concentration, greatly stimulated phosphorylation coupled with oxidation of succinate in rat liver mitochondria, as described previously (*1*).

2. In a hypotonic or alkaline medium in which the mitochomdria are inclined to be unstable the stimulatory effect of the detergent on oxidative phosphorylation is greater.

3. Quatamin 24 P did not stimulate oxidative phosphorylation in aged mitochondria but accelerated decrease in the activity.

4. The rate at which oxidative phosphorylation decreased was also accelerated by preincubation with the detergent.

5. It is posturated that in intact mitochondria the oxidative phosphorylation system is partially inhibited by some factor. This masking factor is removed by the distortion produced by Quatamin 24 P. Therefore under conditions where insignificant inhibition is produced by the detergent an overall stimula-

tion is observed.

6. The detergent stimulated both oxidation of succinate and coupled phosphorylation. The distorted state of the mitochondria caused by the Quatamin 24 P was unstable. The distortion caused by long preincubation of mitochondria with the detergent resulted in great decrease in oxidative phosphorylation.

The author wishes to express his sincere thanks to Prof. K. Okunuki, Mr. K. Iwasa and T. Higashiyama for their kind advice on during this work, and also to Kao Soap Co., Ltd. for generous gifts of detergents.

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EXPERIMENTS ON THE SPECIFICITY OF PANCREATIC RIBONUCLEASE

II. BEHAVIOUR OF THE ENZYME TOWARDS RIBO-APYRIMIDINIC ACIDS*

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The specificity of beef pancreatic ribonuclease (ribonuclease I), which had been crystallized by Kunitz (*2*) in 1940, was studied by Loring (*3*), Schmidt (*4*), Volkin and Cohn (*5*), and especially by Todd *et al.* (*6–8*), and by Markham *et al.* (*9–11*). They proved that the enzyme catalysed the cleavage of internucleotide linkages in ribonucleic acid between the 3′-pyrimidine nucleoside phosphoryl groups and the 5′-hydroxyl groups of the adjacent purine or pyrimidine nucleotide groups, and also that it gave 3′-pyrimidine nucleotides or oligonucleotides bearing terminal 3′-pyrimidine nucleotides, pyrimidine nucleoside 2′:3′-cyclic phosphates being formed as intermediates. However, it was reported by Zamenhof *et al.* (*12*) that a natural polymer of ribose phosphate, which was isolated from *Hemophilus influenzae* type b, was hydrolysed by this enzyme. This seemed to suggest that the presence in ribonucleic acid of the pyrimidines was not necessary for the catalytic activity of this enzyme. There were also reports that the enzyme contains at least two active components, ribonucleases A and B (*13, 14*). Consequently, it has become necessary to reinvestigate the specificity of the enzyme.

To obtain information about the specificity of ribonuclease I, we have studied the behaviour of the enzyme towards derivatives of ribonucleic acid (deaminated (*15*) and methylated (*16*) ribonucleic acid) and towards synthetic substrates (2-hydroxyethyl benzyl phosphate and ethyleneglycol 1,2-cyclic phosphate) (*17*). The present study deals with the behaviour of the enzyme towards ribo-apyrimidinic acids which one of the authors (Takemura) for the first time prepared by hydrazinolysis of yeast ribonucleic acid (*18*).

EXPERIMENTAL

Substrates—Commercial yeast ribonucleic acid (Schwarz Laboratories Inc.) was purified by Sevag's method (*19*), dialysed in the cold against frequent changes of distilled water and lyophilised. Ribo-apyrimidinic acids, Preparations 1, 2 and 3, were described in a previous paper (*18*), where they were listed as RAPYA I, II and IV respectively. Ribo-apyrimidinic acids, Preparations 4, 5 and 6, were obtained by a similar procedure by which RAPYA IV″ was prepared. Yeast ribonucleic acid (1 g.) was dried in a vacuum and treated with anhydrous hydrazine (3 ml.) at 50 to 54° for 60 minutes (Preparations 4 and 5) or at 49 to 51° for 60 minutes (Preparation 6). After the reaction and precipitation with absolute alcohol, the solid materials were dissolved in 100 ml. of phosphate buffer of pH 6.0, dialysed overnight against running tap water, against frequent changes of distilled water for 24 hours and lyophilised. Their base composition is summarised in Table I.

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* Some of this work were presented at the 4th International Congress of Biochemistry in Vienna, September 1–6, 1958 (*1*) and at the 7th Symposium on Nucleic Acids in Nagoya, February 1–2, 1958.

TABLE I

Composition of Nitrogeneous Constituents of the Substrates[1]

Substrate	Adenine	Guanine	Cytidylic acid	Uridylic acid
RNA[2]	10.0	12.6	11.5	10.2
RAPYA,[2] Perparation 1	10.0	12.4	9.7	7.2
2	10.0	12.3	6.2	3.7
3	10.0	12.6	0.6	0.7
4	10.0	10.5	<0.5	<0.7
5	10.0	9.8	1.0	1.0
6	10.0	12.0	1.2	1.8

1) These values are moles of the nitrogeneous constituents per 10 moles of adenine. 2) RNA=yeast ribonucleic acid; RAPYA=ribo-apyrimidinic acid.

Pancreatic Ribonuclease (*ribonuclease I*)—Crystalline ribonuclease I from Worthington Biochemical Sales Co. was used. In experiments where preparations 4, 5 and 6 were employed as the substrates, ribonucleases A and B, active components of the commercial ribonuclease I, were also used. The fractionation of ribonuclease I followed Hirs, Moore, and Stein's procedure (*13*) (Fig. 1). Each eluted fraction corresponding to ribonuclease A (major component) and ribonuclease B (minor component) was collected separately, concentrated by adsorbing on bufferised Amberlite XE-64 (pH 5.38) and eluting with 0.2 *M* phosphate buffer (pH 8). The concentrated solutions were brought to neutral pH and stored in a refrigerator.

Method—Experiments were carried out in buffers of optimum pH for the enzymes. In control experiments the enzymes were omitted. The rates of hydrolysis were measured as described below. After appropriate times of incubation, 0.2 volume of 0.75 per cent uranyl acetate solution in 25 per cent perchloric acid was added to 1 volume portions of the reaction mixtures and of the controls, shaken, stood for 30 minutes at room temperature and centrifuged. The acid soluble hydrolysis products in the supernatants served for the determination of phosphorus (Allen's method (*20*)), and of the intensity of ultraviolet absorption at 260 mμ.. Since the uranyl reagent precipitates ribonucleic acid most effectively from solutions containing *ca.* 3 mg. per ml., this concentration of substrates was used in most experiments. The uranyl reagent does not precipitate mononucleotides and oligonucleotides of low molecular weight. When concentrated solutions of substrates were used, aliquots were diluted with water to make the indicated concentration prior to the addition of the uranyl reagent.

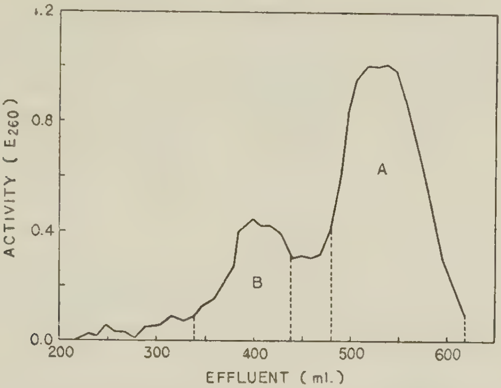


FIG. 1. Fractionation of commercial crystlline ribonuclease I (40 mg.). Exchanger: Amberlite XE-64 bufferized with 0.2 *M* phosphate buffer (pH 6.49), 100×4.1 cm. Eluting solution: 0.2 *M* phosphate buffer (pH 6.49), 1.2 ml./min.. Activities of each fraction were determined by incubating with yeast ribonucleic acid and by measuring the ultraviolet absorption intensities at 260 mμ. of acid soluble hydrolysis products.

Action of Ribonuclease I on the Substrates of Different Pyrimidine Content—60 mg. portions of the ribo-apyrimidinic acids, Preparations 1, 2 and 3, in 4 ml. of 0.2 *M* tris (hydroxymethyl) amino methane buffer (pH 7.6) were incubated with 800 μg. of ribonuclease I at 37°. The acid soluble hydrolysis products were measured by phosphorus determination. The results are shown in Fig. 2.

Action of Various Concentrations of Ribonuclease I on the Ribo-apyrimidinic Acid, Preparation 3—The solutions of ribonuclease I in amounts of 1, 4, 50 and 500 per cent. of the substrate weight were added to 0.35 per cent. solutions of Preparation 3 in 0.2 *M* tris (hydroxymethyl) amino methane buffer (pH 7.6). The experiments were carried out at 0°, since Preparation 3 was unstable in this buffer at 37° and this nonenzymatic degradation was greatly reduced at 0° (*18*) whilst the enzyme is still considerably active. After the final incubation time, total ultraviolet absorption at 260 mμ. of each digested and control solution was measured. The rates of hydrolysis were measured by the determination of absorption intensities of the acid soluble hydrolysis products and plotted as per cent of total absorption (Fig. 3).

Action of Ribonucleases I, A and B on the Ribonucleic Acid and the Ribo-apyrimidinic Acids, Preparations 4, 5 and 6—Stock solutions of ribonucleases A and B were carefully diluted until the activities of 1.5 ml. of the diluted solutions towards ribonucleic acid for 1 hour were equal to that of 1.5 ml. of a solution containing 2 μg. of ribonuclease I. 0.5 ml. of solutions of Preparation 4 and of the ribonucleic acid (6 mg. each) in 0.2 *M* phosphate buffer (pH 7.7) were incubated at 37° with each 1.5 ml. of ribonucleases I (2 μg.), A and B solutions in the same buffer. In experiments using Preparations 5 and 6, 0.2 *M* tris (hydroxymethyl) amino methane buffer (pH 7.6) was used. 1.5 ml. of a mixed solution of ribonucleases A and B was also used. The rates of hydrolysis were measured by the determination of ultraviolet absorption of the acid soluble products and plotted as E_{260}, extinction at 260 mμ., as shown in Fig. 4, 5 and 6.

RESULTS

The ribo-apyrimidinic acids in which the pyrimidines were partially removed from the ribonucleic acid were more or less resistant to the action of ribonuclease I, and as the pyrimidine content of the substrates decreased the rate of hydrolysis was reduced (Fig. 2). Preparation 3, which was practically free from the pyrimidines, was only slightly hydrolysed. As the enzyme concentration increased, however, the rate of hydrolysis of the substrate increased (Fig. 3). When the enzyme in five amounts of the substrate weight was used, the ultraviolet absorption intensity of the acid soluble degradation products reached about 40 per cent. of total intensity. These results seem to show that the presence of the pyrimidines in ribonucleic acid is not necessary for the action of commercial ribonuclease I, or that the hydrolysis is caused by a small amount of a nonspecific phosphodiesterase or a specific diesterase which hydrolyse purine nucleoside phosphodiester groups. The fractionation of the commercial ribonuclease I was therefore carried out in order to clarify these points.

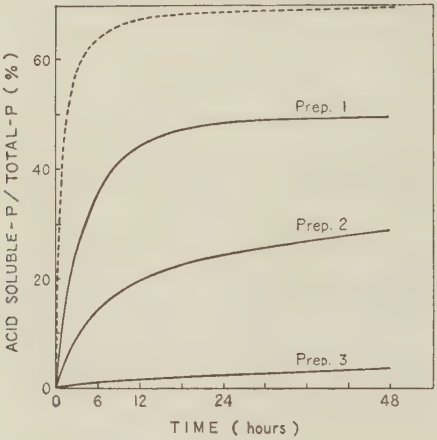


FIG. 2. Action of pancreatic ribonuclease on yeast ribo-apyrimidinic acids of different pyrimidine content. Enzyme/substrate=1.3/100, pH 7.6, 37°.
---- ribonucleic acid, —— ribo-apyrimidinic acids.

As shown in Fig. 4, ribo-apyrimidinic acid 4 was hydrolysed by commercial ribonuclease I, although the rate of the hydrolysis was very slow. On the other hand, the substrate was, within experimental error, not hydrolysed to form acid soluble products either by ribonuclease A or by ribonuclease B. In addition, the activity of ribonuclease I towards ribo-apyrimidinic acids was not recovered by mixing the components, ribonucleases A and B (Figs. 5 and 6). This can also be seen in the cases where ribonucleic acid was used

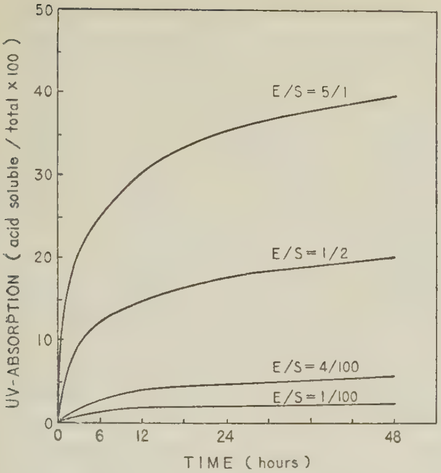


FIG. 3. Action of pancreatic ribonuclease on yeast ribo-apyrimidinic acid 3 in various concentrations of the enzyme. pH 7.6, 37°. E/S=enzyme/substrate.

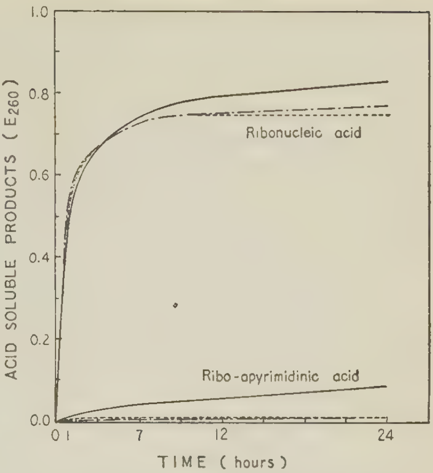


FIG. 4. Action of ribonucleases I, A and B on yeast ribo-apyrimidinic acid 4 and on its parent ribonucleic acid. —— ribonuclease I, - - - - ribonuclease A, —·— ribonuclease B.

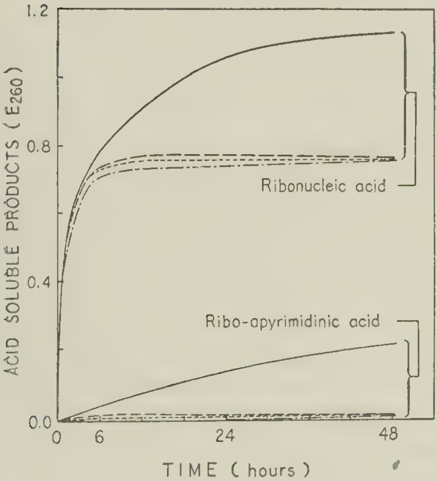


FIG. 5. Action of ribonucleases I, A, B and A+B on yeast ribo-apyrimidinic acid 5 and on its parent ribonucleic acid. —— ribonuclease I, - - - - ribonuclease A, —·— ribonuclease B, — — ribonuclease A+B.

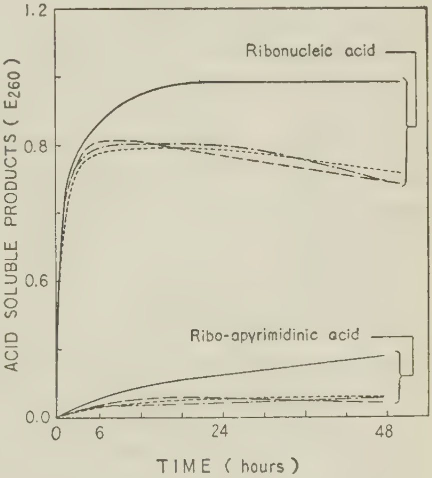


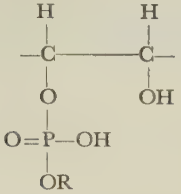
FIG. 6. Action of ribonucleases I, A, B and A+B on yeast ribo-apyrimidinic acid 6 and on its parent ribonucleic acid. —— ribonuclease I, - - - - ribonuclease A, —·— ribonuclease B, — — ribonuclease A+B.

as a substrate. Ribonucleases A and B were used in amounts equal to that of ribonuclease I on the basis of activity towards the ribonucleic acid. Then the rates of hydrolysis of the ribonucleic acid by these enzymes were, at the initial stage, comparable with each other. Whereas, as the incubation continued, ribonuclease I gradually slowly hydrolysed the nucleic acid, even after the hydrolysis either by ribonuclease A or by ribonuclease B, or by their mixture, A+B, had ceased (Figs. 4, 5 and 6). Therefore, the hydrolysis of the riboapyrimidinic acid by ribonuclease I is most probably attributed to the action of a nonspecific phosphodiesterase or of a ribonuclease, specific to purine nucleotide esters, contained in the commercial specimen together with the ribonucleases A and B. The substrate which retained a small amount of the pyrimidines was hydrolysed by each of ribonucleases A, B and their mixture (A+B), although the rates of hydrolysis were slower than that by ribonuclease I (Fig. 6).

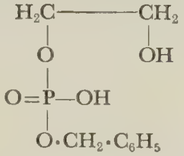
DISCUSSION

The first approach of our laboratory to the problem on the specificity of pancreatic ribonuclease (ribonuclease I) was the study of the action of the enzyme on yeast deaminoribonucleic acid (*15*). The derivative was hydrolysed by this enzyme. The same result was obtained by Vandendriessche (*21*). On the other hand, methylated ribonucleic acid of yeast was completely unhydrolysed (*16*). These results show that the presence in ribonucleic acid of the amino groups attached to bases is not essential but the C2′-hydroxyl groups are essential. Since Zamenhof *et al.* (*12*) reported that a polymer of ribose-3-phosphate was hydrolysed by ribonuclease I, it has been presumed that even the presence of pyrimidines in ribonucleic acid is not a specific requirement for the catalytic activity of this enzyme. In addition, apurinic acid in which a deoxyribonucleic acid was deprived of its purine constituents was hydrolysed by this enzyme (*22*). This substrate has the structural requirements for the mechanism of the action of the enzyme by which cyclic phosphates are formed as the intermediates; *i.e.*, it has the 4′-hydroxyl groups in the adjacent position to the corresponding 3′- or 5′-phosphoryl groups of the internucleotide bonds. Consequently, it has been supposed that the specificity of the enzyme is due to a specific resistance of the purine nucleotide groups against the action of the enzyme rather than to a specific importance of the pyrimidine groups for the catalytic activity of the enzyme (*23*), or in other words, a factor which decide the specificity of ribonuclease I is not the presence of the pyrimidines but the absence of the purines (*5, 24*).

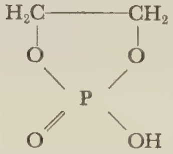
From the facts mentioned above, one of the authors (Egami) proposed, as a working hypothesis, the minimal structural requirements for the action of ribonuclease I, these were the phosphodiester group and the hydroxyl group adjacent to each other (*24*). Hence, 2-hydroxyethyl benzyl phosphoric acid and ethylene glycol-1,2-cyclic phosphate, the simplest substrates for the enzyme, were synthesised in our laboratory, but were not hydrolysed nor inhibited the hydrolysis of ribonucleic acid by the enzyme (*17*). Moreover, the enzyme was inactive towards the P esters of α-glycerophosphoric acid such as L-α-glycerophosphorylcholine and L-α-glycerophosphorylethanolamine (*21*). Accordingly, a compound having a more complicated structure than the above was required as the substrate. The removal of the pyrimidines from ribonucleic acid was then undertaken.



$$-\mathrm{CH(O{-}P(=O)(OH){-}OR){-}CH(OH)}-$$



$$\mathrm{H_2C(O{-}P(=O)(OH){-}O{\cdot}CH_2{\cdot}C_6H_5){-}CH_2(OH)}$$



From the present data, it appears that the presence of the pyrimidines is not essential for the action of commercial ribonuclease I, although the pyrimidines have a certain relation to the enzyme activity (Figs. 2 and 3). However, it can be seen in Figs. 4, 5 and 6 that the pyrimidines participate in the catalytic activities of each ribonucleases A and B which are main components in the commercial ribonuclease I. According to Hakim (*14*), the specificity usually attributed to crystalline ribonuclease I is practically the properties of ribonuclease A (major component), whilst ribonuclease B produces substantially guanosine-3′-phosphate from ribonucleic acid. In spite of this difference in their specificities, both of these enzymes require the presence of the pyrimidines for their catalytic activities. Therefore, the hydrolysis by ribonuclease I of the ribo-apyrimidinic acids almost entirely free from the pyrimidines is attributed to the action of an active minor component other than ribonucleases A and B. It has been said that commercial crystalline ribonuclease I usually contains a nonspecific phosphodiesterase. Besides, recent studies of Hakim (*25*) have demonstrated that crystalline ribonuclease I contains four active components specific to each of the purine and pyrimidine nucleotide esters. Consequently, the minor component which hydrolyses ribo-apyrimidinic acid is probably a nonspecific phosphodiesterase or a ribonuclease which specifically hydrolyses the purine nucleotide esters.

The resistance of the ribo-apyrimidinic acid the enzymic hydrolysis is not due to a nitrogeneous contaminant, which is only slightly if at all present, as described in the preceding paper (*18*). Moreover, no appreciable inhibition of the action of ribonuclease I on the ribonucleic acid was observed either by hydrazine or by ribo-apyrimidinic acid (Table II). It is possible that thd pyrimidines may be responsible for the affinity between ribonucleic acid and each of ribonucleases A and B, although this cannot be concluded unless more strict inhibition experiments by ribo-apyrimidinic acid are performed. If combination with the enzymes is necessary only at the C2′-hydroxyl group and the C3′-phosphodiester group for enzyme activity, it should also be possible for the enzyme to combine with a 3′-hydroxyl and a 2′-phosphodiester group and still be active. If the combination with the enzymes at three points (a point on pyrimidine base, 2′-OH and 3′-phosphate) or more is required for the enzyme activity, the enzymes cannot now combine in such a way to hydrolyse the 2′-5′ internucleotide linkage. Michelson (*26*) reported that the 2′-5′

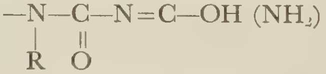
internucleotide linkages in synthetic polyuridylic acid, which contains 3′–5′ and 2′–5′ internucleotide linkages, were unhydrolysed by ribonuclease I. This coincides with our results which prove the necessity of the presence of the pyrimidines. According to Ukita and Irie *(27)* β-methyl riboside 2,3-cyclic phosphate is not hydrolysed by ribonuclease I. This shows that methyl group is not a satisfactory substitute for the pyrimidines.

TABLE II

Effect of Hydrazine and of Ribo-apyrimidinic Acid on the Hydrolysis of Ribonucleic Acid by Ribonuclease I

	Amounts of hydrolysis of ribonucleic acid*		
	2 hrs.	24 hrs.	48 hrs.
RNA, 7 mg./2 ml. (*ca.* 10^{-2} M P.)	100%	100%	100%
RNA, 10^{-2} M P.			
+Hydrazine, 5×10^{-2} *M*	96	100	—
+ ,, , 1×10^{-2} *M*	96	103	—
+ ,, , 1×10^{-3} *M*	100	105	—
RNA, 7 mg.			
+RAPYA, prep. 3, 2 mg.	94	—	106
+ ,, , 4 mg.	95	—	98

* These values are shown as per cent of hydrolysis of ribonucleic acid in the absence of hydrazine or of ribo-apyrimidinic acid, Preparation 3, at each reaction times.

As mentioned above, the pyrimidines in ribonucleic acid participate in the action of ribonucleases A and B. However, it should be emphasised that this does not necessarily mean the necessity of the whole structure of the pyrimidines but of at least one group in the pyrimidines. Witzel *(28)* reported that ribonuclease degraded 4,5-dihydropyrimidine riboside-3′-phosphodiester, but not 3′-phosphodiester of ribose or of ureidopropionyl riboside. Then he suggested that  $-\underset{\displaystyle R}{\underset{|}{N}}-\underset{\displaystyle O}{\underset{\|}{C}}-N=C-OH\ (NH_2)$ group in the pyrimidine ring must be intact for the degradation of the diester by ribonuclease. His opinion also agrees with our results.

SUMMARY

1. The behaviour of commercial crystalline ribonuclease (ribonuclease I, Worthington Biochemical Sales Co.), of ribonucleases A and B, which were obtained by the fractionation of the commercial specimen by Hirs, Moore and Stein's method, towards yeast ribonucleic acid and the ribo-apyrimidinic acids prepared from yeast ribonucleic acid were studied.

2. Commercial ribonuclease I hydrolysed ribo-apyrimidinic acid, although the rate of hydrolysis of the acid was far slower than that of ribonucleic acid.

3. The ribo-apyrimidinic acid was practically unhydrolysed by ribo-

nucleases A, B and their mixture. As the pyrimidine content of the substrates decreased the resistance of the substrates against the action of the enzyme increased.

4. Commercial ribonuclease I probably contains, in addition to ribonucleases A and B, a small amount of a ribonuclease, which hydrolyses purine nucleotide esters, or of a nonspecific phosphodiesterase.

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ACTION OF TRYPSIN AND PAPAIN ON DERIVATIVES OF α-AMINO-γ-GUANIDINOBUTYRIC ACID AND HOMOARGININE*

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It has been known that trypsin hydrolyzes the peptide bonds which contain a carbonyl group of arginine or lysine, as in benzoyl-L-argininamide (*1*). Trypsin can also hydrolyze ester bonds, as in benzoylarginine ester (*1*). Papain hydrolyzes many types of synthetic peptide derivatives, among which benzoylarginine amide or ester is the most sensitive substrate for the enzyme (*2*, *3*).

In the present investigation, an attempt was made to define the effect of changes in the straight-chain length between the terminal guanidino group and the α-carbon atom in benzoylarginine amide or ester on the rate of action by acetyltrypsin, trypsin and papain. Amides and esters of α-benzoylamino-γ-guanidinobutyric acid and benzoylhomoarginine were prepared and subjected to the action of the enzymes.

EXPERIMENTAL

Enzyme

Acetyltrypsin was lyophilized sample (ATR 5603). Crystalline trypsin was the same material as that previously described (*4*). Papain was lyophilized, crystalline sample (2 times recrystallized; PAP 5431). These were the preparations of Worthington Biochemical Corp., U.S.A. The purity of papain was tested by its activity towards benzoyl-L-argininamide (L-BzArgAm). At 39° and pH 6.0 (M/15 phosphate buffer) in the presence of 0.005 M L-cysteine and 0.001 M Versene, the enzyme hydrolyzed 0.05 M L-BzArgAm with a proteolytic coefficient of 0.047**. The values of 1.0∼1.2 (*2*, *5*, *6*) were reported in the literatures.

Methods

The procedure was the same as that described before (*7*). The hydrolysis rates

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[*] The present paper was read partly before the 31st Annual Meeting of the Japanese Biochemical Society (Sapporo, July, 1958).

[**] The authors were informed recently by Dr. C. C. Worthington of Worthington Biochem. Corp. that lyophilization of crystalline papain had a deleterious effect on the enzyme and the Corporation are supplying the crystalline suspension in cysteine solution.

were followed by means of the Conway microdiffusion method for the amide substrates and Hestrin method for the ester substrates. In most cases, the reactions were run at 30° and in 0.01 M initial substrate concentration. The hydrolysis of the substrates tested followed first order kinetics within the limits of experimental error. Proteolytic coefficients (C) were estimated from $C=k/e$, where $k=(1/\text{min.})\log(100/100-$ % hydrolysis) and e is the protein concentration in mg. of N per ml.

Synthesis of Substrates

The general procedure as described by Bergmann *et al.* (*8*) for the preparation of L-BzArgAm was used. The yield of L-α-benzoylamino-γ-guanidinobutyramide hydrochloride which was prepared by amidation of benzoylaminoguanidinobutyric acid ethyl ester hydrochloride was so poor as 12 per cent. In this case, an unidentified crystalline compound which was more soluble than benzoylaminoguanidinobutyramide hydrochloride was produced by a side reaction. N^α-Benzoyl-L-homoarginine could not be obtained in crystalline form in spite of every efforts; neither benzoylation of L-homoarginine (*9*, *10*) nor guanidination of the ε-amino group of N^α-benzoyl-L-lysine (*11*) by treatment with *S*-methylisothiourea gave crystalline product. However, the crystalline racemic benzoylhomoarginine and its amide hydrochloride could be obtained easily as described below.

L-*α-Benzoylamino-γ-guanidinobutyric Acid Ethyl Ester Hydrochloride* (L-*BzAgbaOEt HCl*)-L-α-Benzoylamino-γ-guanidinobutyric acid (13.2 g.) (*12*) was suspended in ethanol (260 ml.) and dry HCl was introduced to saturation at room temperature. The solution was refluxed for 30 minutes and evaporated *in vacuo*. The residue was dissolved in ethanol, and the same procedure was repeated again. Repeated addition and evaporation *in vacuo* of ethanol followed by treatment of the residue with ether yielded crystalline product. This was recrystallized from ethanol-ether; yield, 15 g. (94 per cent); m.p. 203-205° (decomp.); $[\alpha]_D^{10}-48.0°$ (c 1, water).

$C_{14}H_{21}O_3N_4Cl$ (328.8): Calcd. C 51.1, H 6.4, N 17.0
Found C 51.1, H 6.3, N 17.2

L-*α-Benzoylamino-γ-guanidinobutyramide Hydrochloride* (L-*BzAgbaAm HCl*) -The above ester (3.3 g.) was dissolved in methanol (60 ml.) previously saturated with dry NH_3 at 0°, and the solution was kept at room temperature for 2 days. The solution was then concentrated *in vacuo* to dryness, and the product was recrystallized 2∼3 times from water-ethanol; Yield, 0.36 g. (12 per cent); m.p. 258-259° (decomp.); $[\alpha]_D^{14}+65.0°$ (c 1, in water). Paper chromatography in *n*-butanol-acetic acid-water (4 : 1 : 2, *v/v*) yielded one spot (R_f 0.75).

$C_{12}H_{18}O_2N_5Cl$ (299.8): Calcd. C 48.1, H 6.1, N 23.4
Found C 47.9, H 6.0, N 23.8

L-*BzArgOMe* H_2CO_3—This was prepared as described by McDonald and Balls (*3*).

L-*BzArgAm HCl*—This was prepared as described in the literatures (*8*, *13*) with minor modification. After the oily benzoylarginine ethyl or methyl ester hydrochloride was amidated in the usual manner (*8*), the solution was concentrated to dryness *in vacuo*, and the residue including some NH_4Cl as impurity was dissolved in methanol containing a small amount of triethylamine. Repeated addition and evaporation *in vacuo* of methanol and triethylamine until free of NH_3 left crystalline product*. This was recrystallized from hot 95 per cent ethanol. Thus, the yield and purity of the compound were improved.

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* This procedure replaced NH_4Cl with triethylamine hydrochloride which is soluble in ethanol.

Benzoyl-DL-*arginine*—Benzoyl-L-arginine (13.9 g.) (*8*) was treated with glacial acetic acid (40 ml.) and acetic anhydride (3.1 ml.) by the same procedure as that for the racemization of benzoyl-L-histidine (*14*). The product obtained was dissolved in dilute HCl, and the solution was neutralized with dilute NH_4OH to yield a pure crystalline benzoylarginine which possessed no observable optical rotation. Yield, 12.9 g. (93 per cent); m.p. 290–292° (decomp).

$C_{13}H_{18}O_3N_4$ (278.3): Calcd. N 20.1
Found N 20.2

DL-*BzArgAm HCl*—The above compound (5.6 g.) was esterified with ethanol in the same way as that for L-BzAgbaOEt HCl. The oily benzoylarginine ethyl ester hydrochloride which could not be crystallized was amidated in the usual manner. After the solution was concentrated, the syrup was taken up in water and treated with Ag_2O (1.2∼1.8 g.) under cooling. The filtrate was evaporated *in vacuo* until free of NH_3, the residue was dissolved in water, and the pH of the solution was adjusted to 7.5 by the addition of dilute HCl. Removal of the solvent and treatment of the resulting syrup with ethanol and acetone yielded crystals, which were recrystallized from hot ethanol-acetone. This was more soluble in ethanol, less in water and less crystallizable than L-form. Yield, 4.1 g. (61 per cent); m.p. 109–110°.

$C_{13}H_{20}O_2N_5Cl \cdot H_2O$ (331.8): Calcd. C 47.1, H 6.6, N 21.1
Found C 47.8, H 6.7, N 21.6

The compound lost 5.9 per cent of its weight upon drying for 6 hours *in vacuo* at 100° (calculated for $C_{13}H_{20}O_2N_5Cl \cdot H_2O$, H_2O 5.4 per cent).

Benzoyl-DL-*homoarginine*—This was prepared from DL-homoarginine nitrate (12.6 g.) (*9*) in the same way as that for benzoyl-L-arginine (*8*). The product was recrystallized from hot water; yield, 11.8 g. (81 per cent); m.p. 267–269° (decomp).

$C_{14}H_{20}O_3N_4$ (292.3): Calcd. N 19.2
Found N 19.1

Benzoyl-DL-*homoargininamide Hydrochloride* (DL-*BzHmargAm HCl*)—This was prepared from the above compound (5.8 g.) in the same way as that for L-BzArgAm HCl. The product was recrystallized from hot 95 per cent ethanol. Yield, 3.5 g. (48 per cent); m.p. 88–98°.

$C_{14}H_{22}O_2N_5Cl \cdot 2H_2O$ (363.9): Calcd. C 46.2, H 7.2, N 19.3
Found C 45.8, H 7.3, N 19.8

Since the intermediate DL-BzHmargOEt HCl could not be crystallized, non-crystalline powder of it was used in this study.

RESULTS AND DISCUSSION

Action of Acetyltrypsin—Since an experiment concerning the optimum pH with an amide substrate had not been reported, the optimum pH for acetyltrypsin was determined using L-BzArgAm as a substrate, and the results were shown in Fig. 1, an optimum pH appearing near 8.5. As seen from Fig. 1, the pH-activity curves were dependent upon the nature of the buffers used, though the reason could not be clarified. It was reported that the optimum pH of L-BzArgOEt was 8.3 (*15*).

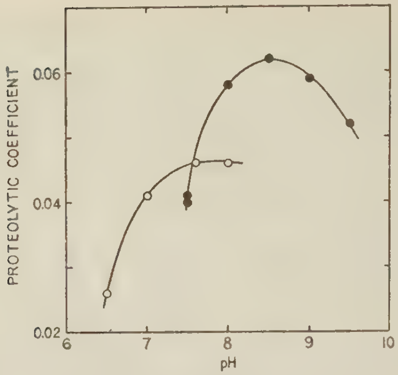

FIG. 1. The pH dependence of the hydrolysis of L-BzArgAm (0.03 *M*) by acetyltrypsin at 30°. ○, 0.1 *M* phosphate buffer. ●, 0.1 *M* with respect to the amine component of Tris-HCl buffer.

As shown in Table I, acetyltrypsin has no detectable action on the homologous series of BzArgAm, despite the fact that L-BzArgAm is a sensitive substrate for the enzyme. Therefore, it is evident that the susceptibility of the substrates is strongly dependent on their straight-chain length between the terminal guanidino group and the α-carbon atom. It is of interest to note that D-BzArgAm exhibits an inhibitory effect on the hydrolysis of L-form (Table I), per cent inhibition ($[(C-C_i)/C]\times 100$) (*16*) being 55, where C_i is the proteolytic coefficient, at the same initial substrate concentration, in the presence of the inhibitor. It may be expected that L-form of BzHmargAm was inert for the enzyme, though its D-form might exhibit an inhibitory effect to some extent.

TABLE I

Hydrolysis of the Substrates by Acetyltrypsin

Substrate concentration, 0.01 *M* with respect to the L-isomer; pH 8.5 (0.1 *M* Tris buffer); temperature 30°.

Substrate	Enzyme concentration (*mg. protein N per ml.*)	*C*	Relative rate
L-BzAgbaAm	0.92	0 1)	0
L-BzArgAm	0.0505	0.21	100
DL-BzArgAm	,,	0.094	45
DL-BzHmargAm	0.92	0 1)	0

1) No detectable hydrolysis in 2 hours.

Action of Trypsin—It is of interest that the homologous derivatives of arginine and ε-aminocaproic acid ethyl ester are hydrolyzed slowly by trypsin (Table II). It appears that α-amino-γ-guanidinobutyric acid derivatives are hydrolyzed more rapidly at 0.01 *M* initial substrate concentration than homoarginine derivatives, but a definite conclusion can not be drawn because D-homoarginine derivatives may have some inhibitory effect on the hydrolysis of L-derivatives. As shown in Table II and III, the hydrolysis of L-BzArgAm was inhibited by the presence of D-BzArgAm, L-BzAgbaAm and BzHmargAm.

TABLE II

Hydrolysis of the Substrates by Trypsin

Substrate concentration, 0.01 *M*; pH 7.8 (0.1 *M* phosphate buffer); temperature, 30°.

Substrate	Enzyme concentration (*mg. protein N per ml.*)	*C*	Relative rate
L-BzAgbaAm	0.50	0.0013	0.7
L-BzArgAm	0.051	0.20	100
DL-BzArgAm	,,	0.12	60
DL-BzHmargAm	1.00	0.0008	0.4
L-BzAgbaOEt	0.0104	2.6	10
L-BzArgOMe	0.00052	25	100
DL-BzHmargOEt	0.052	0.33	1.3
ε-Aminocaproic acid ethyl ester1)	0.0105	0.45	1.8

1) Its hydrochloride was prepared as described in the literature (*19*).

TABLE III

Inhibition of Trypsin by BzAgbaAm and BzHmargAm

Substrate concentration, 0.01 *M*; inhibitor concentration, 0.01 *M* with respect to the L-isomer; pH 7.8 (0.1 *M* phosphate buffer); temperature, 30°.

Substrate + inhibitor	Enzyme concentration (*mg. protein N per ml.*)	*C*	Relative rate
L-BzArgAm	0.052	0.19	100
,, + L-BzAgbaAm	,,	0.15	79
,, + DL-BzHmargAm	,,	0.048	25

The specificity of trypsin appears to be narrower than that of the other proteolytic enzymes considered so far, and a few related compounds with arginine or lysine such as N^{α}-hydroxy-N^{δ}-guanidinovaleric acid ester and several poly-ε-aminocaproyl-α-amino acids are hydrolyzed slowly by the enzyme (*1*, *17*). The homoarginine and α-amino-γ-guanidinobutyric acid derivatives listed in Table II may be taken as the additional related substrates hydrolyzed poorly by the enzyme.

The results in Table II also show that the susceptibility of the substrates in dependent on their straight-chain length as described in the case of acetyltrypsin*. It would be interesting in this connection to note that Hofstee has reported that, concerning the hydrolysis of *n*-fatty acid esters of *m*-hydroxybenzoic acid, trypsin displays sharp optima of activity with respect to the number of C-atoms in the *n*-acyl moiety of the substrate; caproyl derivative (C_6) is hydrolyzed at a maximum rate at high substrate concentrations, C_5 or C_7 compound also being hydrolyzed to some extent (*18*).

Action of Papain—The results shown in Table IV indicate that papain readily hydrolyzes all the substrates tested. The order of susceptibility to hydrolysis at 0.01 *M* initial substrate concentration is listed as: L-BzHmargAm > L-BzArgAm > L-BzAgbaAm. The per cent inhibition by D-BzArgAm on the hydrolysis of the L-form is 43 (Table IV), while the inhibitory effect by D-BzHmargAm on the hydrolysis of L-BzHmargAm can not be determined. Although BzHmargAm appears more sensitive than BzArgAm, the kinetic constants K_m and k_3 should be determined using derivatives of L-homoarginine, L-arginine, and more highly purified preparation of papain for the rational comparison of the susceptibility of substrates.

TABLE IV

Hydrolysis of the Substrates by Papain

Substrate concentration, 0.01 *M*; pH 6.0 (0.05 *M* phosphate buffer) with 0.005 *M* cysteine and 0.001 *M* Versene; temperature, 30°.

Substrate	Enzyme concentration (*mg. protein N per ml.*)	*C*	Relative rate
L-BzAgbaAm	0.151	0.013	22
L-BzArgAm	0.050	0.058	100
DL-BzArgAm	0.10	0.033	57
DL-BzHmargAm	0.075	0.062	107
L-BzAgbaOEt	0.102	0.057	10
L-BzArgOMe	0.051	0.55	100

SUMMARY

1. The amides of L-α-benzoylamino-γ-guanidinobutyric acid, N^α-benzoyl-L- and -DL-arginine, and -DL-homoarginine, and the several ester derivatives have been synthesized and tested as substrates for certain proteolytic enzymes.

2. Acetyltrypsin hydrolyzes benzoylargininamide. No hydrolysis of the amides of α-benzoylamino-γ-guanidinobutyric acid and benzoylhomoarginine was detected.

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* It was also shown that specificity of arginase is somewhat similar to that of acetyltrypsin or trypsin. Partially purified arginase hydrolyzed 0.01 *M* L-α-amino-γ-guanidinobutyric acid at 0 per cent and 0.01 *M* L-homoarginine at about 0.5 per cent, respectively, of the rate of L-arginine (Kitagawa, K., and Izumiya, N., unpublished data).

3. Crystalline trypsin hydrolyzes slowly the compounds of α-amino-γ-guanidinobutyric acid and homoarginine, *e.g.* L-α-benzoylamino-γ-guanidinobutyramide was split at 0.007 times the rate of benzoyl-L-argininamide.
4. Papain hydrolyzes all amide and ester substrates tested.

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IN VIVO INCORPORATION OF S^{35}-SULFATE INTO CHARONINSULFURIC ACIDS

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(Received for publication, January 28, 1959)

In vivo incorporation of S^{35}-sulfate into charoninsulfuric acid (*2*) in the mucous gland of "*Charonia lampas*" (a marine gastropod) was demonstrated by Suzuki (*1*). He observed that the radioactivity of charoninsulfuric acid increased for 69 hours after intramuscular injection of S^{35}-sulfate.

In this paper *in vivo* incorporation of S^{35}-sulfate into charoninsulfuric acids with different contents of sulfur (one being s-rich fraction and the other s-poor fraction) is presented.

EXPERIMENTAL

Separation of S^{35}-Labeled Charoninsulfuric Acids and Inorganic Sulfate from the Mucous Glands—1.7 mc./15 ml. water of S^{35}-sulfate (carrier free) were injected into mucous gland of six groups of "*Charonia lampas*", each group comprising five animals. The animals of each group kept living in the sea were killed individually at each different time after the injection. The mucous glands were removed and immediately immersed in absolute ethanol. Separation of S^{35}-labeled charoninsulfuric acids and inorganic sulfate was done as shown in Scheme 1.

The possibility that the samples of charoninsulfuric acids thus obtained might have been contaminated with free S^{35}-sulfate need not be considered because the control (at time 0) treated by the same procedure never gave the labeled sulfate.

Determination of Radioactivity—The weight of charoninsulfuric acids (s-rich and s-poor) of each group thus obtained was found to be approximately 800 mg. and 350 mg. respectively. The radioactivity of both charoninsulfuric acids was measured with the gas flow counter at infinite thickness (24.5 mg. per cm^2). The weight of barium sulfate of each group so obtained was about 50 mg., and the radioactivity of the barium sulfate was measured at a thickness of about 10 mg. per cm^2 under the end-window Geiger counter.

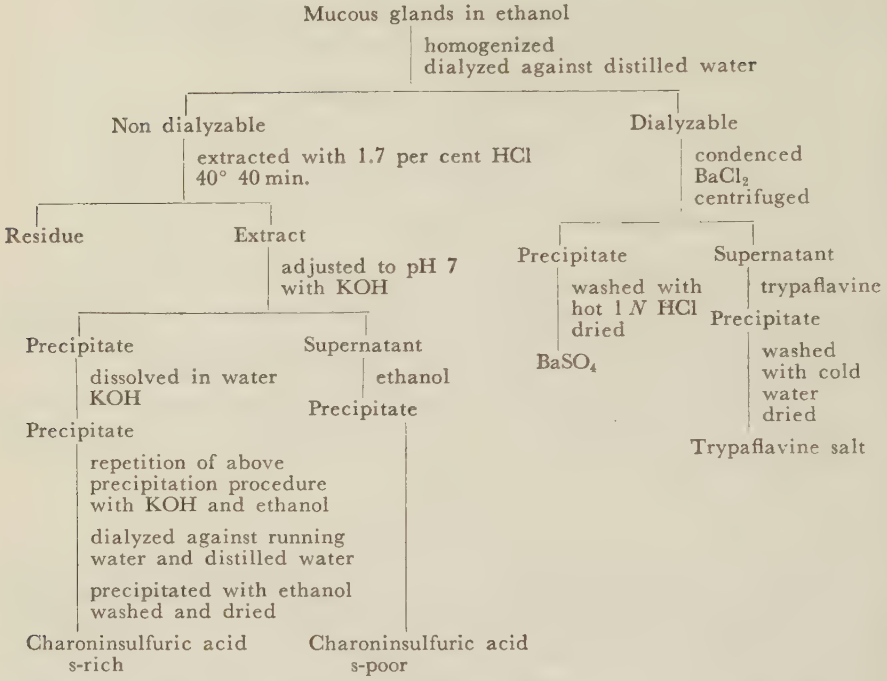
Thus the weight of each sample measured having been different (24.5 mg. per cm^2, or about 10 mg. per cm^2), all the values of radioactivity were converged into those values where the thickness was extremely thin, in such a way that they were the thickness was extremely thin, in such a way that they were calibrated by calibration coefficient against self-absorption in each thickness.

RESULTS AND DISCUSSION

As can be seen in Table I, in both of the two charoninsulfuric acids, the radioactivity increased independently on the sulfate content till the expiration

SCHEME 1

Fractionation of S^{35}-labeled Mucous Glands



of 96 hours after the injection and thereafter decreased rapidly. If the incorporation of S^{35} was due to a mere exchange of S^{35} and S only, the relative radioactivity (c.p.m./per cent S) of s-poor and s-rich fraction should have been the same. But as will be shown in the Fig. 1, the relative radioactivity (c.p.m./per cent S) of s-poor fraction was found rather more than of the s-rich one. This fact disproved the above assumption (Fig. 1). These findings lead to the conclusion that *in vivo* incorporation of S^{35}-sulfate into charoninsulfuric acids is not a mere exchange of S^{35} and S in the charoninsulfuric acids only, but it is mainly used for the esterification of free hydroxyl groups in both charoninsulfuric acids.

While the radioactivity of barium sulfate decreased till the expiration of 96 hours after the injection, and thereafter for about 72 hours no decrease was observed.

All the precipitates with trypaflavine showed no appreciable radioactivity.

SUMMARY

1. 1.7 mc. of S^{35}-sulfate were injected into mucous gland of thirty "*Charonia lampas*" (a marine gastropod). S^{35}-labeled mucous glands removed at different time were fractionated and the radioactivity of each fraction was

TABLE I

The Radioactivity of Charoninsulfuric Acids

Time (*hours*)	S content (%)	Total radioactivity (*c.p.m./dish*)	Relative radioactivity	
			(*c.p.m./%s*)	(*c.p.m./glucose*)
S-rich fraction				
3	14.5	0	0	0
18	13.6	32.0	2.35	68.5
48	14.0	211	15.0	452
72	14.9	631	42.3	1350
96	15.7	840	53.5	1800
120	14.9	158	10.6	339
S-poor fraction				
3	0.785	0	0	0
18	0.910	33.7	37.0	34.2
48	0.830	63.2	76.2	64.0
72	0.641	152	240	154
96	0.616	170	276	172
120	0.644	58.4	90.7	59.2

1.7 mc. of S^{35}-sulfate were injected into mucous gland of thirty "Charonia". At different time, mucous glands were removed and charoninsulfuric acids were separated.

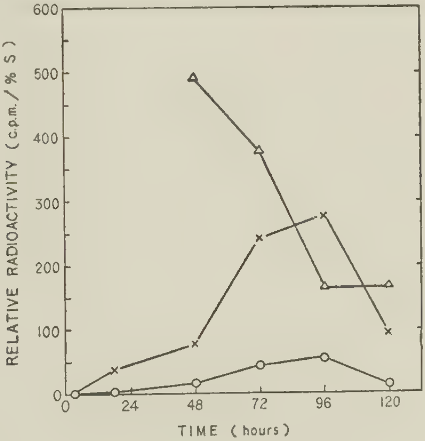


FIG. 1. The relative radioactivity of charoninsulfuric acids and barium sulfate of mucous gland.

1.7 mc. of S^{35}-sulfate were injected into mucous gland of thirty "*Charonia lampas*". S^{35}-labeled mucous glands removed at different time were fractionated and the radioactivity of charoninsulfuric acids and inorganic sulfate was measured.

—○— s-rich fraction; —×— s-poor fraction; —△— inorganic sulfate.

measured.

2. The radioactivity of both charoninsulfuric acids (s-rich and s-poor fraction) increased independently on the sulfur content till the expiration of 96 hours after the injection and thereafter decreased rapidly.

3. *In vivo* incorporation of S^{35}-sulfate into charoninsulfuric acids is not a mere exchange of S^{35} and S in the charoninsulfuric acids only, but it is mainly used for the esterification of free hydroxyl groups in both charoninsulfuric acids.

4. The radioactivity of inorganic sulfate decreased till the expiration of 96 hours after the injection and thereafter for about 72 hours no decrease was observed.

I wish to express my thanks to Prof. F. Egami for his kind encouragement and interest. The pxpense of this study was defrayed in part by a grant to Prof. F. Egami from the Ministry of Education. This study was supported by Seikagaku-Kenkyujo Ltd. too, to which the author's thanks are presented. A part of the experiment was executed in the Marine Biological Laboratry of Nagoya University.

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SUR LA POLYMERISATION ET LA FORME MOLECULAIRE DE LA GLUTAMICO-DESHYDROGENASE

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(Mémoire reçu, le 30 janvier, 1959)

Deux méthodes permettent la préparation de la glutamico-déshydrogénase active à l'état de cristal et de pureté très avancée: l'une est due à M.M Olson et Anfinsen (*1*), l'autre à M. Strecker (*2*). On a déjà remarqué dans l'activité enzymatique la non-identité de ces deux préparations. Olson *et al.* ont montré par la sédimentation, la diffusion et la pycnométrie, que le poids moléculaire de la glutamico-déshydrogénase de foie de boeuf est d'un million. Ce poids moléculaire est remarquablement plus grand que celui des autres déshydrogénases.

Nous avons récemment mis au point une méthode modifiée par laquelle la glutamico-déshydrogénase de foie de boeuf, de porc ou d'homme est cristallisée très facilement avec un bon rendement (*3*) et nous avons caractérisé les propriétés physico-chimiques de cette protéine enzymatique, dans le but d'étudier le mécanisme de l'action de l'enzyme.

Nous nous sommes demandés d'abord pourquoi le poids moléculaire tellement grand a été obtenu. On a révélé dans notre travail suivant qu'une molécule de cet enzyme se compose au moins de trois sous-unités actives. Ensuite nous avons vérifié, de point de vue thérmodynamique, un type de combinaison entre le coenzyme et l'enzyme.

MATERIEL ET METHODE

Glutamico-Déshydrogénase—Les auteurs ont préalablement observé que certains sels sont capables de protéger l'activité de glutamico-déshydrogénase contre la chaleur, surtout le sulfate de sodium est le plus efficace (la Fig. 1). Parce que cet enzyme ne se trouve que dans la mitochondrie, comme M. Hogeboom l'a démontré autrefois, afin de l'extraire dans la solution, il est nécessaire que la membrane de mitochondrie soit détruite d'avance. Alors nous y avons appliqué les deux moyens physiques; soit la chaleur à 58°, soit la haute pression osmotique par le sulfate de sodium à 5 pour cent. Au cours du franctionnement, le pH de la solution de l'enzyme a été maintenu auprès de 6.4 où l'enzyme est le plus stable.

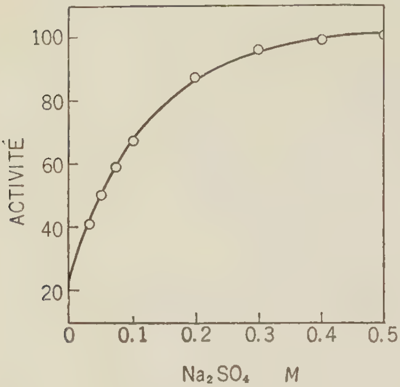


FIG. 1. Stabilisation de l'activité d'enzyme par le sulfate de sodium.

Protéine d'enzyme est dissoute dans la solution du tampon de phosphate (*M*/10, pH 6.4) contenant de sulfate de sodium. La solution est réstée pendant 1 heure à 50°

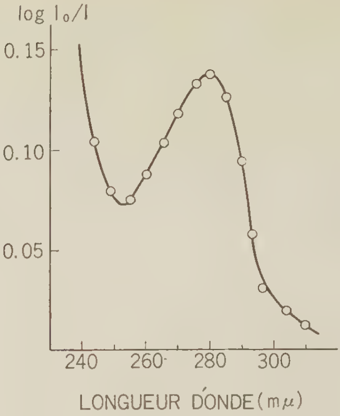


FIG. 2. Spectre de l'absorption à la lumière ultraviolette de la glutamico-déshydrogénase extraite du foie de boeuf. Concentration de l'enzyme : 0.14 mg./ml. Solvant : Solution de tampon phosphaté 0.1 *M*, pH 7.6

Nous avons cristallisé la glutamico-déshydrogénase selon le schéma.

La quantité de la protéine enzymatique a été évaluée à la densité optique mesurée au spectrophotométrie de Beckman, à 280 mμ, en prenant comme coefficient, la valeur 1.03 pour 1 mg./cm³.

Le dosage de la déshydrogénase s'effectue à 25° en ajoutant 0.05 ml de la solution d'enzyme à une solution qui se compose de glutamiate 13 mM, DPN 150 μM et du tampon phosphaté (0.1 *M*, pH 7.6), dont le volume final atteint 1.5 ml.

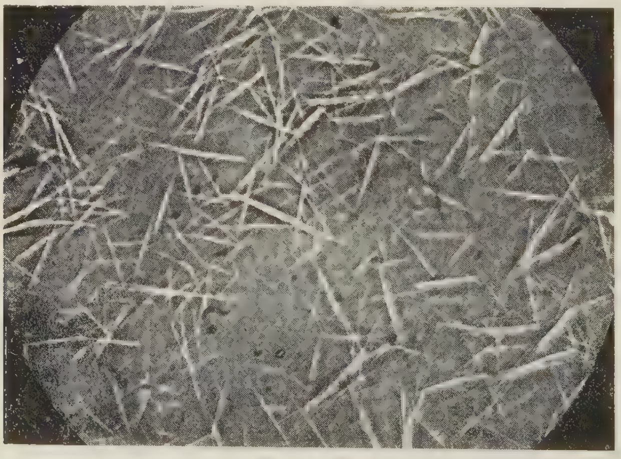
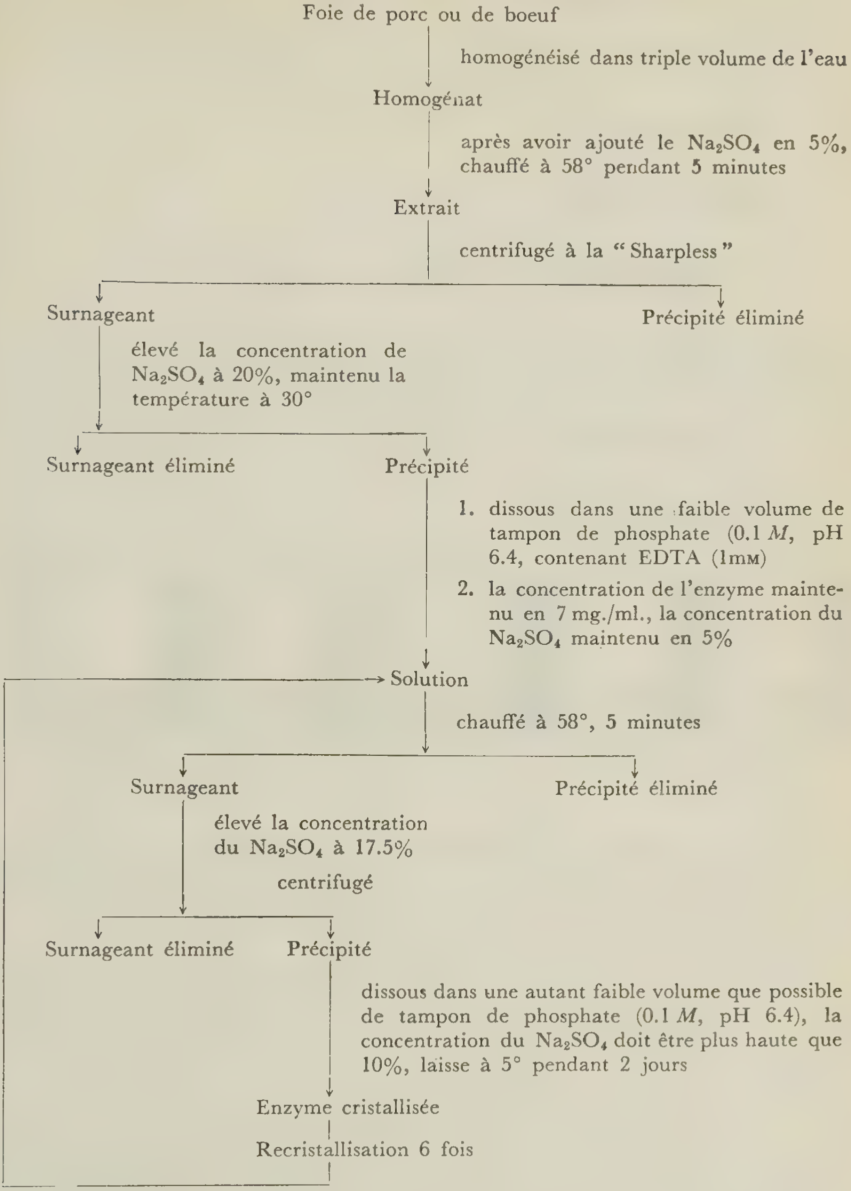


FIG. 3. Image microscopique des cristaux de la glutamico-déshydrogénase de foie d'homme.

Schéma de la cristallisation de la glutamico-déshydrogénase



Foie de porc ou de boeuf
homogénéisé dans triple volume de l'eau
Homogénat
après avoir ajouté le Na_2SO_4 en 5%, chauffé à 58° pendant 5 minutes
Extrait
centrifugé à la "Sharpless"
Surnageant
Précipité éliminé
élevé la concentration de Na_2SO_4 à 20%, maintenu la température à 30°
Surnageant éliminé
Précipité
1. dissous dans une faible volume de tampon de phosphate (0.1 *M*, pH 6.4, contenant EDTA (1mM)
2. la concentration de l'enzyme maintenu en 7 mg./ml., la concentration du Na_2SO_4 maintenu en 5%

Solution
chauffé à 58°, 5 minutes
Surnageant
Précipité éliminé
élevé la concentration du Na_2SO_4 à 17.5%
centrifugé
Surnageant éliminé
Précipité
dissous dans une autant faible volume que possible de tampon de phosphate (0.1 *M*, pH 6.4), la concentration du Na_2SO_4 doit être plus haute que 10%, laisse à 5° pendant 2 jours
Enzyme cristallisée
Recristallisation 6 fois

Quant à l'unité d'activité enzymatique, si l'on définit qu'une unité correspond à la quantité d'enzyme qui donne une variation de 0.001 en densité optique à 340 mμ par minute, la glutamico-déshydrogénase purifiée a 15,000 unités pour mg. Cette valeur est de 30 pour cent plus grande que celle rapportée par M. Strecker *et al.* La figure 2 montre le spectre d'absorption de notre enzyme.

Nous n'y avons reconnu aucun nucléotide combiné que M. Strecker avait observé dans son enzyme.

L'homogénéité de l'enzyme a été vérifiée par l'électrophorèse et l'ultracentrifuge (les Fig. 4 et Fig. 5).

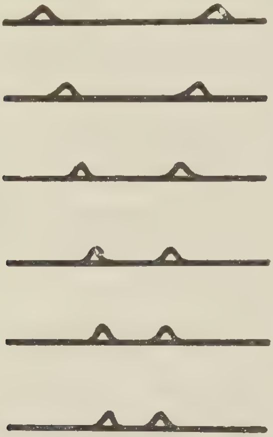


Fig. 4. Image de l'électrophorèse de la glutamico-déshydrogénase de foie de porc. Enzyme dialysé pendant 24 heures contre la solution de tampon phosphaté 0.1 *M*, pH 7.6

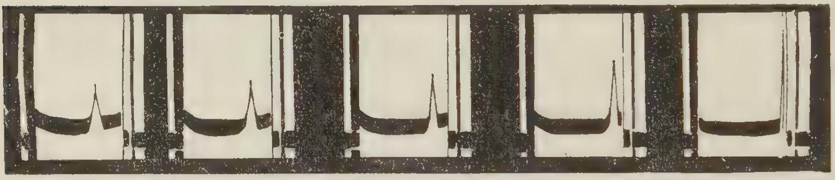


Fig. 5. Ultracentrifugation de la déhydrogénase glutamique de porc concentration de protéine 10 mg./ml., 59800 t.p.m. intervalle 480″, $\theta=75$, $S=23\cdot10^{-13}$.

Protéinase cristallisée de *B. subtilis* offerte par le Laboratoire de M. Okunuki.

La déshydrogénase de D-glycéraldéhyde-3-phosphate a été cristallisée par la méthode de M. et Mme Cori (*4*). Nous en avons utilisée après l'avoir récristallisée 3 fois. Une molécule de cette enzyme contient deux ou trois molécules de DPN combinés.

Ovoalbumine d'oeuf: matériel commercial.

DPN extrait de la levure de boulangerie et purifié par des échangeurs d'ions (Doulite C 10 d'abord, ensuite Dowex 1). Sa pureté a été de 100 pour cent, calculée à l'aide de la densité optique à 340 mμ, prenant comme coefficient le chiffre de $6.22\cdot10^6$ cm^2/mole (*5*).

DPNH: On ajoute l'alcool jusqu'à sa concentration finale soit de 10 pour cent et l'alcool déshydrogénase de la levure 1 mg./ml. à une solution de DPN (100 mg./ml.) amené à pH 9.5. Au cours de la réaction, le pH de la solution a été controlé à pH 9.5 à l'aide de l'électrode en verre, en ajoutant une faible quantité d'une solution de soude (0.01 *N*). Au bout de 5 minutes la solution a été amenée à pH 10, chauffée à 100° pendant 3 minutes pour dénaturer la protéine. Après avoir ajouté l'alcool à 50 pour cent, le précipité a été élimine par centrifugation. Au surnageant obtenu, on a ajouté 5 fois de volume de l'alcool absolu et après contrifugation, le précipité a été lavé avec certaine quantité de l'alcool absolu et seché sous vide (pureté 100 pour cent).

L'urée et le dioxane: Matériaux commerciaux.

Diffusion de la lumière de la protéine enzymatique éffectuée par le photomètre de Phoenix-Brice. Incrément spécifique d'index réfractif déterminé par le réfractomètre différentiel de Phoenix.

Sédimentation de la protéine mesurée par l'ultracentrifugueuse de Spinco model E. Electrophorèse effectuée par l'appareil de Hitachi.

RÉSULTATS

1. *Détermination du Poids Moléculaire de la Glutamico-Déshydrogénase au Moyen de la Diffusion de la Lumière*

A. *Théorie* (5) (6)—Dans le cas de la solution non-idéale où les mouvements thermiques des particles ne sont pas mutuellement indépendants, l'intensité de la lumière diffusée par la solution n'est plus représentée comme une somme de diffusion de chaque soluté; elle se diminue moins que celle de la solution idéale, à cause de l'interférence mutuelle. Dans ce cas, la fluctuation de la distribution locale du soluté a pour résultat de provoquer l'héterogénéité locale de l'index réfractif de la solution, par laquelle la diffusion de la lumière soit observée. D'après cette considération, M. Smolcowsky a conduit la formule suivante

$$R_{90} = \mathrm{K}\frac{RTC}{\partial P/\partial C} \qquad (1)$$

R_{90}: Intensité réduite de la lumière diffusée en sens de 90° par le soluté
R: Constante de gaz
T: Température absolue
P: Pression osmotique de la solution
C: Concentration de la solution

Quant à la pression osmotique de la solution diluée, la formule suivante est donnée

$$\frac{P}{RT} = \frac{C}{\mathrm{M}} + BC^2 \qquad (2)$$

M: Poids moléculaire du soluté
B: Coéfficient du virial secondaire

Portons la formule (1) dans la formule (2)

$$\frac{KC}{R_{90}} = \frac{1}{\mathrm{M}} + 2BC$$

l'autre part, on a la relation suivante ente l'intensité réduite R_{90} et le turbidité τ

$$\tau = \frac{16}{3}\pi \mathrm{R}_{90}$$

donc,

$$\frac{HC}{\tau} = \frac{1}{\mathrm{M}} + 2BC$$

où

$$H = \frac{32\pi^2 RT}{3\lambda^4} n_0^2 \frac{(n-n_0)^2}{C^2} \qquad (3)$$

Dans le cas de la solution de la protéine où la concentration en sel est assez grande, la constante B approche en général à zèro, on obtient

$$\lim_{c\to 0} \frac{HC}{\tau} = \frac{1}{\mathrm{M}} \qquad (4)$$

B. *Expérience*—La solution a été préalablement centrifugée sous 40 à 60 mille *g* pendant une heure, pour éliminer des poussières, ensuite filtrée diréctement dans une cuvette au moyen d'un "membrane filter" No. 5 de Goettingen. La turbidité, c'est la différence entre la turbidité mesurée de la solution et celle du milieu. Au-dessus de la concentration en protéine de 100μg/ml, la solution concentrée a été diluée successivement, alors qu'au-dessous de 100μg/ml, on a ajouté certaine quantité de protéine dans la solution du tampon.

La Fig. 6 montre l'incrément d'index réfractif en fonction de la quantité de glutamico-déshydrogénase. Par là on obtient la valeur

$$dn/dC = 0.185 \text{ à } 546\ \mathrm{m}\mu.$$

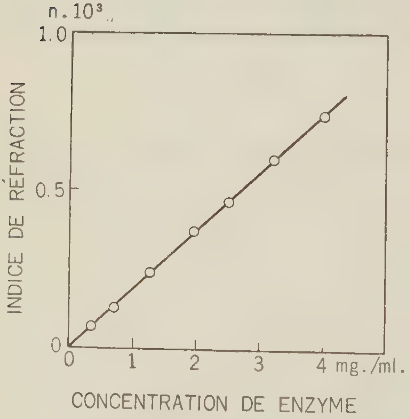


FIG. 6. Gradient de l'indice de réfraction de la solution de glutamiço-déshydrogénase de foie de porc pour sa concentration, *dn/dC*.

Protéine dialysée pendant 20 heures à 5° dans la solution de tampon de phosphate (*M*/30, pH 6.4).

$\lambda = 546\ \mathrm{m}\mu$, $dn/dC = 0.185$

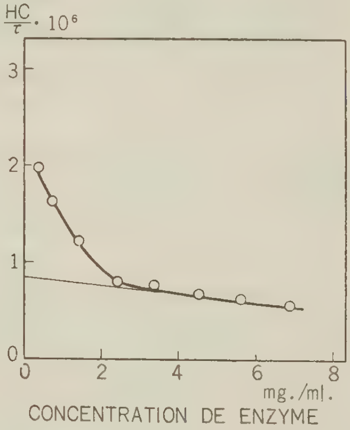


FIG. 7. Diffusion de la lumière de la glutamico-déshydrogénase de foie de porc en concentration dense.

Tampon de phosphate (*M*/10, pH 6.4)

$\lambda = 546\ \mathrm{m}\mu$

Les Figures 6—10 montrent HC/τ en fonction de C.

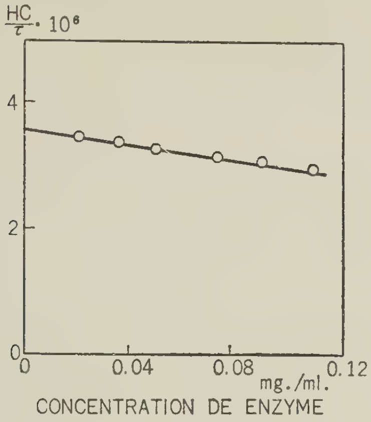


FIG. 8. Diffusion de la lumière de la glutamico-déshydrogénase de foie de porc la concentration diluée.

Tampon de phosphate (M/10, pH 6.4)

$\lambda = 546$ mμ

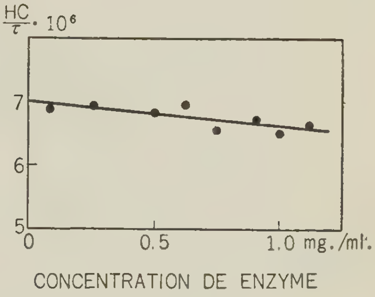


FIG. 9. Diffusion de la lumière de la triose phosphate déshyrogénase.

Tampon de phosphate (M/10, pH 7.6)

$\lambda = 546$ mμ

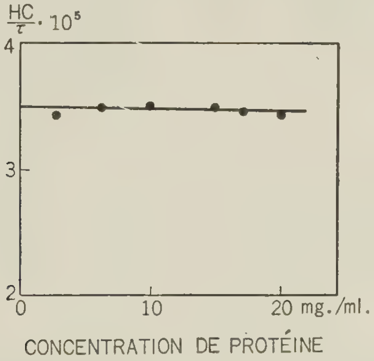


FIG. 10. Diffusion de la lumière de la protéine de *Bacillus subtilis*.

Tampon de phosphate (M/20, pH 7.5)

$\lambda = 546$ mμ

Il est remarquable que la relation entre HC/τ et C dévie de la forme ordinaire, de manière que la constante B a un coefficient négatif et se varie en fonction de la concentration de la protéine. Il est donc possible que les molécules de la protéine enzymatique en effet ont une forte interaction mutuelle.

Dans la région de la concentration de protéine de 2 à 7 mg./ml., le coefficient B a une valeur relativement petite, donc le poids moléculaire de cet enzyme est capable d'être un million par l'extrapolation. Cepandant dans la région où la concentration de la protéine est au-dessous de 1 mg./ml., B a une plus grande valeur négative, mais au-dessous 0.1 mg./ml., HC/τ converge à une valeur, ce qui montre que le poids moléculaire est de 350 mille.

2. *Forme de la Glutamico-Déshydrogénase, Déduite de la Dissymétrie Angulaire de la Diffusion de la Lumière*

Si la longueur d'une molécule de soluté est plus grande qu'un dixième de la longueur d'onde lumineuse appliquée, la distribution angulaire de la diffusion n'est plus symétrique, à cause de l'interférence intérieure. Alors, l'intensité réduite R (θ) est donné par la formule suivante, introduisant

$$\lim_{c \to o} \mathrm{R}(\theta) = \mathrm{K}CM(1+\cos^2\theta)\mathrm{P}(\theta)$$

P (θ) est decidé selon la forme de la molécule; sphère, bâtonnet ou cylindre.

L'intensité réduite en sens 45° et celle en sens 135°, elles sont représentées par R_{45} et R_{135}. On appelle Z $(=\mathrm{R}_{45}/\mathrm{R}_{135})$ le rapport dissymétrique.

Nous avons mesuré R_{45} et R_{135} de la solution d'enzyme en utilisant une cuvette sémioctagonale. Le Tableau I montre la relation entre C et Z.

TABLEAU I

Dissymétrie Moléculaire de la Glutamico-Déshydrogénase

Concentration de glutamico-déshydrogénase (mg./ml.)	1.2	1.1	0.66	0.59	0.41	0.32	0.25	0.19
Rapport de dissymétrie Z	$1,1_0$	1.1_4	1.1_0	1.1_2	1.1_0	1.0_3	1.0_2	1.0_6

Tampon de phosphate $M/10$, pH 6.4

Si la concentration de l'enzyme est au-dessus de 2mg./ml., Z porte une valeur de 1.1.

Il en résulte que si la forme est bâtonnet, L/λ est égale à 0.17, et si elle est sphère, D/λ à 0.1, c'est-à-dire que cet enzyme serait un bâtonnet dont la longueur L est de 700 Å, ou bien une sphère dont le diamètre D est de 400 Å.

On connaît bien dans certaines conditions les formules suivantes qui ont été utilisées pour calculer P (θ) pour la sphère ou le bâtonnet (7).

$$\text{Sphère : } \mathrm{P}(\theta) = \left[\frac{3}{X^3}\,(\sin X - X\cos X)\right]^2,\ X = \frac{2\pi D}{\lambda}\sin\frac{\theta}{2}$$

$$\text{Batonnet : } \mathrm{P}(\theta) = \frac{1}{X}\int_0^{2x}\frac{\sin\omega}{\omega}\,d\omega - \left(\frac{\sin X}{X}\right)^2,\ X = \frac{2\pi L}{\lambda}\sin\frac{\theta}{2}$$

On peut calculer la valeur du poids moléculaire d'après la formule (*4*), en y introduisant la valeur L ou D déjà obtenue dans les formules ci-dessus. En comparant le poids moléculaire calculé de ces deux valeurs-là et en considérant les résultats de la sédimentation, on peut savoir qu'il faut mieux prendre la forme "bâtonnet" pour cette enzyme.

3. Dissociation de la Glutamico-Déshydrogénase en Présence de l'Urée ou de la Dioxane

Comme nous écrirons plus tard, on a observé qu'une molécule de la glutamico-déshydrogénase dont le poids moléculaire est de 350 mille peut combiner certain nombre de DPN et de substrat. Il semble donc que cette petite unité consiste d'une plus petite sous-unité.

Afin de vérifier cette supposition nous avons entrepris deux expériences. Dans la première, certaine quantité de l'urée a été ajoutée à la solution de l'enzyme, où la molécule de l'enzyme se dissocie en quelque sous-unité qui a le poids moléculaire de 70 mille. Mais l'activité enzymatique a été complètement perdue. La figure 11 montre ce résultat.

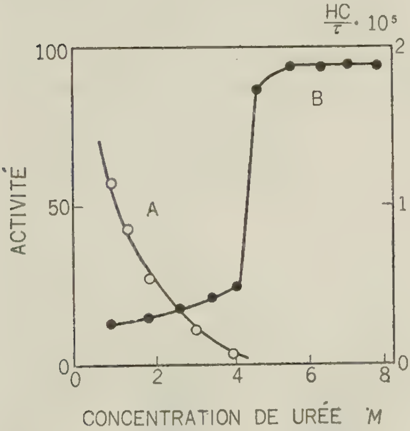


FIG. 11. Glutamico-déshydrogénase déspolymérisée par l'urée et son activité enzymatique.
A : activité enzymatique
B : *HC/τ*

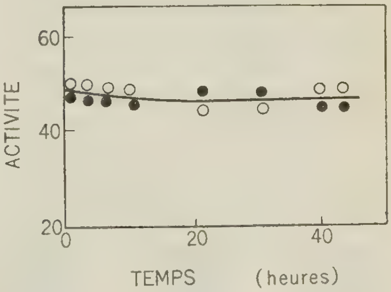


FIG. 12. Influence de la dioxane sur l'activité de la glutamico-déshydrogénase.

Tampon de phosphate (*M*/30, pH 6.4) avec sulfate de sodium (3%). Tampon de phosphate (*M*/30, pH 6.4) avec sulfate de sodium (3%) et dioxane (33.3%)

20°

Dans la seconde, le poids moléculaire a été évalué dans le milieu de dioxane (30 pour cent), la molécule de l'enzyme se dissocie en effet réversiblement en sous-unité sans que l'activité enzymatique soit perdue à 5° pendant 45 heures (la Fig. 12).

Le poids moléculaire de cette sous-unité est évalué de 300 mille ou 140 mille à rapport de la concentration de dioxane de 16.6 pour cent ou 33.3 pour cent (la Fig. 13).

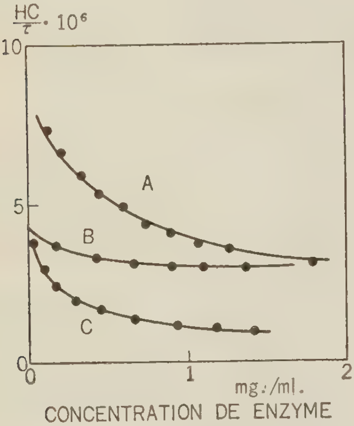


Fig. 13. Dépolymérisation de la glutamico-déshydrogénase par la dioxane.

A: protéine+dioxane (33.3%) +Na_2SO_4 (3%)

B: protéine+dioxane (16.6%) +Na_2SO_4 (3%)

C: (protéine + dioxane de 33.3%) dialysée 20 heures à 5° et isolée de la dioxane

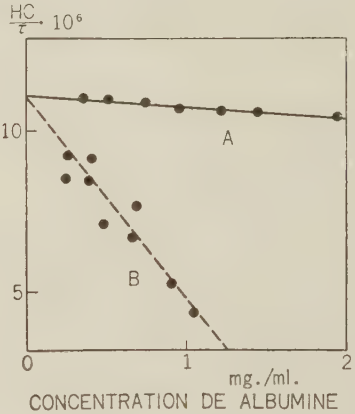


Fig. 14. Diffusion de la lumière de l'ovoalbumine en présence de dioxane.

A: protéine

B: protéine+dioxane (16.6%). Tampon de phosphate (*M*/30, pH 6.4)

La Fig. 14 montre une expérience sur l'albumine de boeuf dans les mêmes conditions expérimentales que celles de la Fig. 13. On n'y trouve

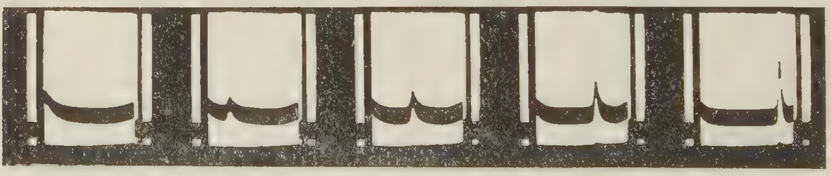


Fig. 15. Image de la sédimentation de la glutamico-déshydrogénase.

Enzyme 6.8 mg./ml. dans la solution de dioxane de 16.5 pour cent mélangée avec le tampon de phosphate de $M/30$ et de pH 6.4, 18° 59780 t.p.m., intervalle 480″, $\theta=55°$, $S=7.1\cdot10^{-13}$.

aucune variation de poids moléculaire, tandis que la constante B a une plus grande valeur négative.

La Fig. 15 montre un diagramme de sédimentation de la glutamico-déshydrogénase en milieu de dioxane de 16.5 pour cent. La constante de sédimentation est réduite en tiers de celle qui a été obtenue en l'absence de dioxane.

4. *Combinaison entre la Glutamico-Déshydrogénase et DPN*

Pour démontrer directement la combinaison entre l'enzyme et le coenzyme, il y a certain nombre de méthodes: ultracentrifugation par Velick, spectroscopie rapide et sensible par Chance et dialyse en équilibre par Klotz, *etc.* Nous avons adopté la méthode d'ultracentrifugation. La solution mélangée de la glutamico-déshydrogénase et DPN est centrifugée sous 190 mille *g* pendant 2 heures par Spinco E. Alors, la protéine est tout à fait précipitée en emportant de DPN fixé et laissant de DPN libre dans le surnageant. Il est possible de calculer la quantité de DPN emporté par la densité optique du surnageant à 260 mμ et la donnée initiale de DPN. Par la formule bien connue (*8*).

$$r/C = kn - kr$$

où

r: Nombre de DPN combiné par une molécule de l'enzyme
n: Valeur maximum
C: Concentration de DPN libre
k: Constante d'association d'enzyme et DPN

En supposant que toutes les combinaisons se fassent sans l'interférence

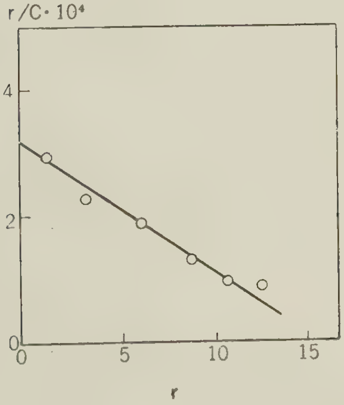


FIG. 16. Nombre de combinaison du DPN avec la glutamico-déshydrogénase.
$n=14\sim15$, $K_n=3.2 \cdot 10^4$

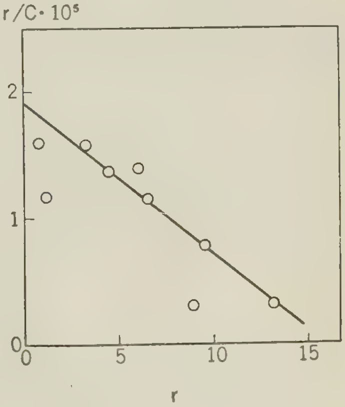


FIG. 17. Nombre de combinaison du DPNH avec la glutamico-déshydrogénase.
$n=15\sim16$, $K_n=1.2 \cdot 10^5$

mutuelle, que toutes les constantes d'association du complexe enzyme-DPN soient égales et que le poids moléculaire de l'enzyme soit un million, nous avons trouvé qu'une molécule de l'enzyme se combine avec environ 15 molécules de DPN. La constante de dissociation du complexe enzyme-DPN est evaluée de 3.10^{-4} M (la Fig. 16).

La même combinaison entre l'enzyme et DPN réduit a été observée. Nous y avons trouvé que n est égale à 15 aussi et la constante de dissociation ($1/k$) à 8.10^{-5} M (la Fig. 17).

DISCUSSION

D'après MM. Olson et Anfinsen nous avons vérifié que le poids moléculaire de la glutamico-déshydrogénase est d'un million dans la même condition où la concentration de l'enzyme mesurée soit entre 2 et 7 mg./ml., mais suivannt une autre méthode, c'est à dire que leur méthode était l'ultracentrifugation, la nôtre la diffusion de la lumière. Ensuite, nous avons mis en évidence qu'à la concentration de l'enzyme diluée au-dessous de 100μg./ml. le poids moléculaire descend au-dessous d'un million et atteint environ 300 mille, c'est à dire, l'unité de la molécule de l'enzyme se dissocie en trois ou quatre sous-unités qui ont éventuellement l'activité enzymatique. Ce fait a été démontré pour la première fois au moyen de la diffusion de la lumière qui est plus sensible que l'ultracentrifugation, spécialement sur la solution diluée de la protéine. Car la diffusion de la lumière est capable de mesurer avec assez de précision le poids moléculaire de la glutamico-déshydrogénase de 10 μg./ml., tandis que la limité méthodique de l'ultracentrifugation est à la concentration de 1 mg./ml.

Les résultats représentés dans les Fig. 7 et Fig. 8 nous conduisent à exposer la relation sur la dissociation de l'enzyme. Représentons une sous-unité de l'enzyme (P.M. 330 mille) et une molécule associée (P.M. 1 million) par E_1 et E_3, on obtient

$$3E_1 = E_3, \qquad K = (E_1)^3/(E_3)$$

Si les concentrations en poids de E_1, E_3 et l'enzyme total sont réspectivement C_1, C_3 et C, C est égale à (C_1+C_3). D'autre part, reprenons le poids moléculaire moyen et celui de E_1 par $\overline{\mathrm{M}}$ et M, alors

$$\overline{\mathrm{M}} = \frac{C_1+3C_3}{C_1+C_3} \cdot \mathrm{M}$$

y mettant la relation $HC/\tau = \overline{\mathrm{M}}$ de la Fig. 7,

$$K = \frac{(3\mathrm{M}-\overline{\mathrm{M}})^3}{3\mathrm{M}_2(\mathrm{M}-\mathrm{M})} \cdot C^2$$

d'après la formule ci-dessus on obtient

$$K = 1.5 \cdot 10^{-11} \quad \text{mole}^2 \cdot l^{-2}$$

$$\Delta G = -RT \ln K = 16\ \text{kcal} \cdot \text{mole}^{-1}$$

Parce qu'il y a deux lieux de la combinaison, auxquels les sous-unités se lient, l'énergie libre pour une liaison serait 8 kcal. pour une mole. Sur la manière de dissociation de l'enzyme, on peut considérer deux possilités, l'une est repésentée par A de la Fig. 18, l'autre par B.

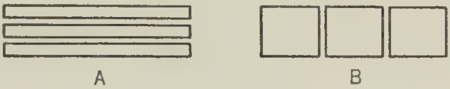


Fig. 18. Schéma de dissociation de l'enzyme polymérisé.

Parmi deux types le schéma *B* est plus vraisemblable parce que nous avons préalablement observé que le rapport de dissymétrie de la diffusion de la lumière, *Z*, converge à la valeur de 1 lorsque la concentration de l'enzyme, *C*, devient très faible.

La diffusion de la lumière, destinée à calculer le poids moléculaire déjà connue, il nous reste à déterminer que la longueur de molécule de soluté, L, est plus ou moins grande qu'un dixième du longueur d'onde lumineuse appliquée. Notre enzyme se situe juste à la limité de cette démarcation, donc nous y soumettons l'équation pour $L < \lambda/10$.

Ainsi, nous avons vu que les molécules de l'enzyme dépolymérisées en trois sous-unìtés conformément à la dilution tiennent l'activité enzymatique. Quand l'enzyme est dissout dans un millieu d'urée, il aussi se dissocie en sous-unité plus petite d'environs 70 mille de poids moléculaire. Cette sous-unité n'a pas d'activité enzymatique du tout. Ce serait l'effet d'urée qui détruit trop de liaison d'hydrogène dans la protéine enzymatique.

Au contraire, quand l'enzyme est dissout dans un millieu de dioxane de 33 pour cent, elle se dissocie en sousunité de 140 mille de poids moléculaire, portant l'activité enzymatique complète. La solution d'enzyme traitée par dioxane se repolymérise après d'être dialysé contre l'eau. Ici, il faut attirer attention à ce que nous avons accompli cette expérience dans le milieu de solvant mixte, où se ferait l'absorption sélective d'un solvant à la surface de l'enzyme. D'après M. Debye, à la limite $C \to o$, on peut appliquer l'équation (3) (9), mettant $(dn/dC + \alpha dn/d\varphi)$ au lieu de dn/dC, où

φ: Volume partiel d'un solvant à l'autre
φ_1: φ à l'état adsorbé à la surface de la protéine
α: $d\varphi^1/dC$

Nous avons mesuré la diffusion de la lumière de l'ovoalbumine dans le milieu de solvant mixte. Comme il est bien coonnue, elle ne se dissocie, ni s'associe. La valeur de $\lim_{c \to o} HC/\tau$ est identique à celle qui a été mesurée dans le milieu du tampon phosphaté, il est permis que α est négligeablement petite.

A partir des résultats d'ultracentrifugation, nous avons calculé l'énergie libre pour combinaison enzyme-DPN, $\Delta G = -4.8$ kcal/mole, ainsi pour la combinaison enzyme-DPNH, $\Delta G = -5.7$ kcal/mole; ces deux valeurs coincident bien avec les valeurs obtenues cinétiquement. On peut en trouver un fondement thérmodynamique pour les résultats cinétiques.

Considérons les faits ci-dessus, nous ferons un schéma pour l'état physique de la glutamico-déshydrogénase (la Fig. 19).

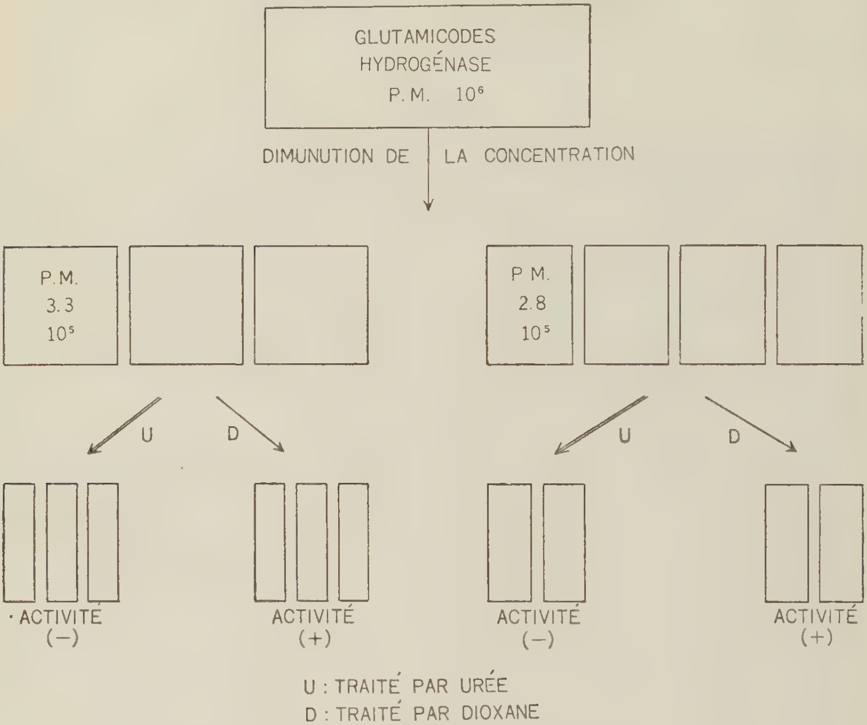


Fig. 19. Schéma de la dépolymérisation de la glutamico-déshydrogénase à cause de dilution de concentration et en ajoutant le dioxane ou l'urée.

RÉSUMÉ

Nous avons modifié la méthode de l'extraction de la glutamico-déshydrogénase qui avait été exposée par M. Olson et M. Strecker. Notre traitement est caractérisé par l'utilisation d'un stablisateur de l'activité d'enzyme (Na_2SO_4), par lequel l'enzyme extrait des foies de boeuf, de porc ou d'homme est cristallisé très faciliment avec un bon rendement.

Quelques propriétés physico-chimiques ont été recherchées.

L'enzyme possède l'activité de 12.10^3 unités pour 1 mg.
Au moyen de la diffusion de la lumière, son poids moléculaire a été évalué

près de 1 million, un résultat qui coincide bien avec la donnée par M. Olson *et al.* Mais, si la concentration de l'enzyme descend au-dessous de 100 μg./ml., le poids moléculaire se réduit à environ $3{\cdot}5{\cdot}10^5$. Il est donc possible que l'enzyme peut être polymérisé si la concentration est grande et qu'il se dissocie à la monomère si la solution est diluée.

Le résultat de la diffusion de la lumière nous montre que la forme moléculaire de l'enzyme peut être "bâtonnet".

Le nombre du locus dans l'enzyme auquel le DPN peut combiner est calculé de 15, ce résultat a été obtenu au moyen de la méthode d'ultracentrifugation.

L'énergie libre pour combinaison de l'enzyme avec DPN ou avec DPNH est évaluée de 4.8 kcal ou 5.7 kcal pour mole respectivement.

Avant de terminer, nous nous permettons de dire que nous avons l'honneur de dédier cet article, pour féliciter la soixante-dixième anniversaire, à Monsieur R. Shôji, professeur honoraire à l'Université de Kioto et à la Faculté de Médecine de Kôbé, un des physiologistes avant-coureurs qui ont introduit la physicochimie dans leur domaine.

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CARBOHYDRATE CHANGES IN THE ACID-SOLUBLE FRACTIONS OF THE CILIATE PROTOZOON TETRAHYMENA GELEII W DURING THE COURSE OF SYNCHRONOUS CULTURE

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In 1955, E. Zeuthen and O. Scherbaum succeeded in synchronizing the cell division of a ciliate protozoon *Tetrahymena pyriformis* (*1*), and this method of experimint opened a new avenue of approach to the biochemical study on the process of cell division in microanimals (*2–6*).

The importance of carbohydrates as energy sources and materials for synthesis of cell constituents is well known. The present investigation was designed to determine the fates of carbohydrates in the mechanism of cell division. Changes in the content of sugars in the acid-soluble fraction of *Tetrahymena geleii* W were examined during the whole course of synchronous culture mainly through color tests and the fractionation procedure of LePage, and some preliminary data have been obtained which showed that some changes in the concentration of acid-soluble sugar compounds took place during the synchronous culture of *T. geleii* W.

MATERIALS AND METHOD

Experimental Organism and Culture Method—*Tetrahymena geleii* W was obtained through the courtesy of Dr. Ishii of the Hosei University. Pure culture of *T. geleii* W was grown in 2 per cent polypepton (Takeda) and 0.5 per cent yeast extract (Takeda) in distilled water. Salts were added as basal medium A of Kidder and Dewey (*7*), except that phosphates were omitted. The medium was filtered and sterilized in an autoclave at 112° for 15 minutes. Stock cultures were maintained in a refrigerator at 2°. Under these conditions the cells could be successfully transplanted after 1 month.

Synchronous Culture—The method used here to synchronize the cells of *T. geleii* W was essentially the same as that employed by Scherbaum and Zeuthen (*1*). One-liter Fernbach culture flasks were used, with each 150 ml. of the culture medium being 15 mm. in depth and 100 cm^2 in area. The inoculated flask was floated on a water bath, the temperature of which was controlled in a manner as illustrated in Fig. 1. The flask was given a horizontal rotatory movement with 60 rotations per minutes and 5 cm. amplitude. The neck of the flask was plugged with cotton. The cell density at the beginning of synchronous culture was disigned to give a population of $10^{4.5-4.7}$ cells per ml., which was taken from the middle logarithmic phase on the growth curve of the cells. The cell number was counted with a hemocytometer of Fuchs-Rosenthal. The division index was calculated as the number of dividing cells over total cells.

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Preparation and Fractionation of the Cells—Approximately 30 ml. of cell suspension was taken from the culture flask at different stages of the synchronous culture. The cells were separated from the suspension by centrifugation at 1,000 r.p.m. for 3 minutes. After washing with ice-cold 0.5 per cent NaCl which was reported to be isotonic with regard to these cells*, the cells were homogenized in 10 per cent cold TCA** by a Potter-Elvehjem homogenizer. The homogenate was centrifuged, and the residue was homogenized again with 1 ml. of 0.5 per cent TCA, and centrifuged. The combined supernatants were brought to a definite volume. This is the acid-soluble fraction.

An aliquot of this fraction was adjusted to pH 8.2, and 1 ml. of 25 per cent barium acetate was added according to the method of LePage (*8*) for the fractionation of acid-soluble phosphate compounds. Four volumes of 95 per cent ethanol was then added and the precipitate was washed once with ethanol. This precipitate is the fraction containing alcohol insoluble matters including water soluble and insoluble barium salt (Ali). The combined supernatant as dried *in vacuo* at room temperature and the residue was dissolved in dilute HCl. This is the alcohol fraction (Als) which contains alcohol-soluble barium salts. Each of the above fractions was freed from barium by the addition of 1 *N* H_2SO_4 followed by centrifugation, and subjected to chemical analysis.

The cold TCA-insoluble residue was extracted with hot 5 per cent TCA for 15 minutes at 100°, washed once with 5 per cent TCA according to the method of Schneider (*9*), and analyzed for its nitrogen by the micro-Kjeldahl method. The combined supernatants were subjected to the nucleic acid determinations. DNA was estimated by the method of Keck (*10*) and RNA, by the orcinol test of Mejbaum (*11*).

The sugar analyses were carried out by the following methods; total sugar by α-naphthol-H_2SO_4 (*12*) and indole-H_2SO_4 (*13*), hexose by primary cysteine-H_2SO_4 (*14*), ketosugar by cysteine-carbazole-H_2SO_4 (*15*), and pentose by orcinol (*11*).

RESULTS AND DISCUSSION

Fig. 1 represents the course of a typical synchronous culture. Synchronous division was obtained by applying eight cycles of temperature changes from 28.5 to 33.7°, and the peak of the division was observed 90 minutes after returning to the optimal temperature. The cell concentration was doubled after the division. These data agreed well with those of Scherbaum and Zeuthen except the time necessary to reach the synchronous division after returning to the optimal temperature, which they reported to be 75 minutes. The yield of the cells was about 500 mg. wet weight per vessel.

As previously demonstrated (*5*), cell substances of the synchronous culture, when measured in terms of dry weight, cell volume, and nitrogen and nucleic acid contents per each cell, increase gradually during the controlled temperature treatment and reach the maximal value before synchronous division. The parallel changes of DNA, RNA and protein nitrogen through the synchronous culture were also noted in strain W. The data in Fig. 2 illustrate that the changes in RNA and DNA contents are in parallel with the change of protein nitrogen. The fact should be emphasized that the majority of cellular components of *T. geleii* increase in quantity at parallel rates during the synchronous culture. However, during the period, a marked change was observed in the amount of acid-soluble sugars which deviated from the parallel change of cell constituents as shown in Fig. 3. The increase in total sugar, hexose, pentose, and ketosugar was greater than expected from the observed increase in other cell substances.

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* Personal communication from Dr. Mita of Hosei University.

** The following abbreviations were used; TCA, trichloroacetic acid; RNA, ribonucleic acid; DNA, deoxyribonucleic acid.

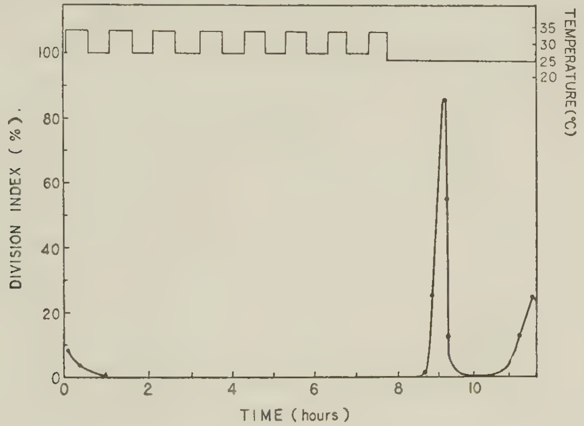

FIG. 1. Synchronous culture of *Tetrahymena geleii* W.

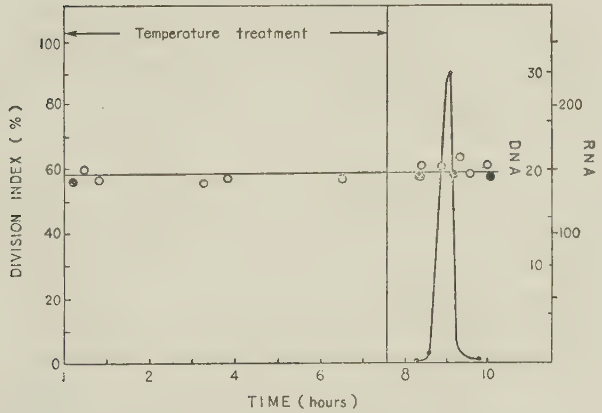

FIG. 2. DNA, RNA and total nitrogen of *T. geleii* W during the synchronous culture. The values of RNA and DNA are expressed as μg. per total nitrogen content (mg. $\times 10^3$).

Over 80 per cent of the total sugars in the acid extract of normal *T. geleii* cells is aldohexose, 10 per cent each or less being pentose and ketosugar. When the controlled temperature changes were applied, total sugar, hexose, pentose, and ketosugar increased gradually to reach the maximal values just before the cell division, and showed a sudden decrease after the division. At the stage just before the synchronous division, over 90 per cent of the acid-soluble sugar was found as hexose, a smaller percentage as pentose and ketosugar.

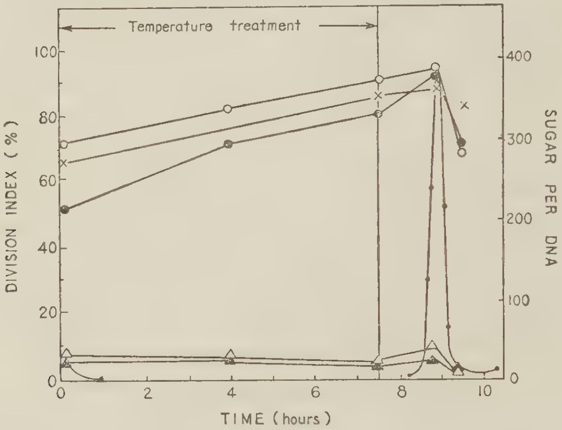

FIG. 3. Variations of total sugar, hexose, pentose, and keto-sugar during the synchronous culture of *T. geleii* W. The values of sugar content are expressed as μg. per optical unit of DNA estimated at 490 mμ. —○—, total sugar by α-naphthol and H_2SO_4; —●—, total sugar by indole-H_2SO_4; —×—, hexose by cysteine-H_2SO_4; —▲—, pentose by orcinol; —△—, ketohexose by cysteine carbazole-H_2SO_4.

The experiments described here were repeated several times in whole or in part, with reproducible results. The absolute amount of sugars in normal cells varied from day to day, but a similar tendency to increase in the amount of sugars was observed with samples taken from the same vessel during the course of synchronous culture.

The fractionation of acid-soluble sugar compounds showed more distinctly the effect of synchronous culture upon *T. geleii* W as shown in Table I. It was observed that most of the total sugar which accumulated with synchronous culture appeared in the fraction containing the alcohol insoluble barium salts (Ali). The maximal concentration was obtained just before the synchronous division, being about twice the value of normal cells. The potential power of reducing which denotes the increase of reducing power after 10 minute hydrolysis in 1 *N* HCl at 100°, showed no appreciable change during the culture. On the other hand, free sugar showing a reducing power without preceding hydrolysis was not detected throughout the experiments. From the data obtained above, it is concluded that the sugar accmulated in the alcohol-insoluble fraction (Ali) was aldohexose and the masking of its reducing site was relatively firm.

Pentose appeared largely in the alcohol-insoluble fraction (Ali), and ketosugar almost evenly in two fractions. Changes in the amount of both these sugars were similar to that of the total sugar throughout the course of synchronous culture.

TABLE I

Variations in the Amount of Sugar Compounds in the Acid-Soluble Fraction of T. geleii W during Synchronous Culture

Fraction	Acid-soluble, alcohol-insoluble fraction			Acid-soluble, alcohol-soluble fraction		
The stage of cells	(1)	(2)	(3)	(1)	(2)	(3)
Total sugar	368	678	594	121	2	17
Reducing sugar	0	0	0	0	0	0
Potential power of reducing	304	278	268	0	0	0
Ketosugar	5	18	16	2	13	16
Pentose	19	26	19	2	1	3

Figures in parentheses denote the stages of cells when the samples were taken. Stage 1 is of cells before the temperature treatment, Stage 2, just before the synchronous division, and Stage 3, just after division. The unit of sugar is μg. per optical unit of DNA.

One-quater of the total sugar in the acid-soluble fraction was found in the alcohol-soluble fraction (Als) and showed a marked decrease in amount during the temperature control. To investigate the nature of this sugar the alcohol-soluble fraction was hydrolyzed in a sealed tube at 100° with 3 N H_2SO_4. The reducing power appeared after 3 hours' heating. During the acid hydrolysis it was noted that the color value in the α-naphthol test (*12*) increased several times. Therefore, the value of total sugar in the alcohol-soluble fraction is far from the true content of the sugar. Evidence for the presence of at least two different kinds of sugar in this fraction could be obtained from the solubility data to ether and color tests for several sugars. No decisive explanation can be given at present about the biological meaning of the change in amount of the sugar in this fraction, until more precise fractionation and analysis are established. However, the fact that the alcohol-soluble sugar decreases very markedly, maintaining the low values through the whole stages of culture and failing to rise after the synchronous division, might be related to a metabolism which is highly susceptible to elevated temperatures. The significance of change in the amount of sugar in the acid-soluble fraction during the course of synchronous culture can not be clarified without isolation and identification of these compounds and knowledge of their metabolism. Recently it was reported that the incorporation of P^{32} into RNA decreased before the cell division of *Tetrahymena* (*3*), with decrease in protein synthesis in the same period[*]. If this is true, it would be reasonable to assume that the acid-soluble sugars which are known to be utilized as materials and energy source for the nucleic acid and protein syntheses are accumulated in the cells as a result of the failure of their utilization in this period.

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[*] Reported at the 59th Meeting of the Kanto Section of the Japanese Biochemical Society in Tokyo, September, 1956, by R. Sugimura, T. Ono, and T. Mita.

SUMMARY

Variation in the amount of acid-soluble sugar compounds was followed during the course of synchronous culture of *Tetrahymena geleii* W mainly by the method of color tests and the fractionation technique of LePage.

During the whole stages, contents of DNA, RNA, and protein nitrogen of a cell change in parallel. Hexose and pentose in the acid-soluble fraction increased during the controlled temperature treatment, reached the maximal values before cell division, and then decreased. The hexose-containing compound in the acid-soluble alcohol-soluble fractions decreased through the whole stages observed.

It was concluded that during the synchronous culture and synchronous division of *Tetrahymena geleii* W, changes in the chemical composition of the acid-soluble sugar compounds took place which were correlated to cytological changes of the cells.

The author express his gratitude to Prof. N. Shimazono for his helpful suggestions and revision of this paper.

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PRECIPITATION OF NUCLEIC ACIDS BY KANAMYCIN AND VIOMYCIN

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(Received for publication, February 7, 1959)

Cohen (*1*, *2*) has described the precipitation of desoxyribonucleic acid (DNA) by streptomycin. This action of streptomycin on DNA and its reversal by salts were confirmed by Berkman *et al.* (*3*), and the influence of certain substances on the activity of streptomycin was further studied by Donovick *et al.* (*4*). Kanamycin is a new antibiotic inhibiting Gram positive and negative bacteria including mycobacteria (*5*). It resembles streptomycin in that it is absorbed to charcoal and eluted in acidmethanol and in that it is insoluble in organic solvents but easily soluble in water. The authors have found that both DNA and ribonucleic acid (RNA) are precipitated by kanamycin and viomycin, and they will report the results in the present paper.

MATERIALS AND METHODS

Sodium desoxyribonucleate (DNA) from sperm (Nutritional Biochemicals Corporation, U.S.A.) and sodium ribonucleate (RNA) from yeast (Wako Pure Chemical Industries, Ltd.) were used throughout the study. Appropriate dilutions were prepared of the nucleic acids and used for experiments.

Dihydrostreptomycin sulfate (manufactured by Meiji Co.), kanamycin sulfate (manufactured by Meiji Co.), and viomycin sulfate (manufactured by Parke & Davis, Co.) were used as streptomycin, kanamycin, and viomycin, respectively.

Sörensen's phosphate buffer solutions consisting of KH_2PO_4-Na_2HPO_4 were added to reaction system so as to give appropriate final concentrations and appropriate pH. If not described especially, the final concentration of phosphate buffer used in the study was $M/60$ and the pH was 6.0.

Reaction tube usually contained 4.0 or 8.0 ml. of reaction system. Reaction system consisted of the following composition: Nucleic acid solution, one part; Kanamycin, viomycin, or streptomycin solution, one part; Phosphate buffer, one part; Distilled water or additional solution of salts, one part.

The turbidity measured by optical density at 610 mμ has been used as an index of the degree of precipitation, although it may be not always an accurate index as seen in Tables 1 to 3 that will be later shown. The measurement was performed by Leitz's Rouy-Photometer, Model LCZ (filter 610 mμ). Readings were made after the reaction system was allowed to stand at room temperature for ten minutes.

A part of this work has been reported in "*Medicine and Biology*" (*in Japanese*), **50** 196-199 (1959), as a preliminary report.

Amounts of DNA were determined by the diphenylamine reaction (*6*) and amounts of RNA were determined by the orcinol-HCl reaction (*7*).

Both nucleic acids supplied as purified forms and nucleic acids extracted from *Mycobacterium avium*, strain Jucho, were also used in the study. Seven-day-old cultures in Sauton medium were used as sources of the extraction made according to Chargaff and Saidel (*8*).

RESULTS

Comparison of Ability between Kanamycin, Viomycin, and Streptomycin to Precipitate Nucleic Acids—As shown in Table I, effects of kanamycin and viomycin on the precipitation of nucleic acids are much more significant than the effect of streptomycin, when observed from the turbidity caused by these antibiotics. It is noteworthy that kanamycin and viomycin precipitate both DNA and RNA, while streptomycin precipitates practically DNA alone.

TABLE I

*Comparative Effects of Streptomycin (SM), Kanamycin (KM), and Viomycin (VM) on Precipitation of Nucleic Acids**

Concentration of antibiotic (*μg./ml.*)		1,000	500	250	125	62.5	0
SM	DNA	0,252	0,125	0,056	0,022	0,000	0,000
	RNA	0,022	0,000	0,000	0,000	0,000	0,000
KM	DNA	0,796	0,770	0,745	0,585	0,061	0,000
	RNA	0,423	0,372	0.276	0,036	0,000	0,000
VM	DNA	0,921	0,620	0,310	0,000	0,000	0,000
	RNA	1,071	0.959	0,310	0,004	0,000	0,000

* The degree of precipitation was expressed as optical density (turbidity) at 610 mμ.

The reaction system consisted of 4.0 ml., and final concentrations of the system were as follows: streptomycin (SM), kanamycin (KM), or viomycin (VM), varied; DNA or RNA, 500 μg./ml.; Phosphate buffer, pH 6.0, *M*/60.

Evidences of Precipitation of Nucleic Acids by Chemical Assay—Chemical assays of DNA and RNA were conducted by the diphenylamine reaction and the orcinol-HCl reaction, respectively. Supernatants obtained by centrifuging were supplied for the assays. The results are shown in Tables 2 to 4. They indicate that precipitation of nucleic acids have really occurred and that kanamycin and viomycin can precipitate nucleic acids much more significantly than streptomycin. Furthermore, they indicate that these antibiotics precipitate both DNA and RNA, unlike the case of streptomycin. It has been demonstrated that about fifty per cent of DNA and RNA are precipitated in the presence of 125 μg. of kanamycin or in the presence of 1,000 μg. of viomycin under the conditions tested (Table II and III).

TABLE II

*Amounts of DNA and of RNA Found after Removal of Precipitates Produced by Kanamycin and Nucleic Acids**

Final concentration of kanamycin (μg/ml.)		1,000	500	250	125	0
DNA in supernatant (μg./ml.)		130	150	175	230	500
RNA in Supernatant (μg./ml.)		90	100	170	280	500
Rate of precipitation	DNA	74%	70%	65%	54%	0%
	RNA	82%	80%	66%	44%	0%

* Reaction system consisted of 8.0 ml, and final concentrations of the system were as follows: Kanamycin, 1,000 μg./ml. to 0 μg./ml.; DNA or RNA, 500 μg./ml.; Phosphate buffer, pH 6.0, $M/60$.

Amounts of DNA and of RNA were determined by diphenylamine reaction and orcinol-HCl reaction, respectively.

TABLE III

*Amounts of DNA and of RNA Found after Removal of Precipitates Produced by Nucleic Acids and Viomycin**

Final concentration of viomycin (μg./ml.)		2,000	1,000	500	250	0
DNA in supernatant (μg./ml.)		200	233	335	465	490
RNA in supernatant (μg./ml.)		80	235	290	480	495
Rate of precipitation	DNA	59%	52%	32%	5%	0%
	RNA	84%	53%	41%	3%	0%

* Reaction system consisted of 8.0 ml, and final concentrations of reaction system were as follows: Viomycin, 2,000 μg./ml. to 0 μg./ml.; DNA or RNA, 500 μg./ml.; Phosphate buffer, pH 6.0, $M/60$.

Amounts of DMA and of RNA were determined by diphenylamine reaction and orcinol-HCl reaction, respectively.

As shown in Table IV, the antibiotics precipitate both nucleic acids purified and nucleic acids extracted from *Mycobacterium avium* as a mixture form.

TABLE IV

Precipitation of Nucleic Acids Extracted from Mycobacterium avium (strain Jncho) by Streptomycin, Kanamycin, and Viomycin[1]

Final concentration of antibiotic	Turbidity before removal of Precipitate	Amounts of nucleic acids found after removal of precipitate by centrifugation	
		DNA[2]	RNA[2]
Nodurg (control)	0.000	25(*μg./ml.*)	480(*μg./ml.*)
Streptomycin, 500 μg./ml.	0.196	65	365
Kanamycin, 500 μg./ml.	0.297	<8	250
Viomycin, 500 μg./ml.	0.347	<8	215

1) Reaction system consisted of the following composition: Nucleic acid solution[3], 4.0 ml.; *M*/15 phosphate buffer, pH 6.0, 2.0 ml; Aq. dest., streptomycin, 2 mg./ml., kanamycin, 2 mg./ml., or viomycin, 2 mg/ml, 2.0 ml.

2) Determined by diphenylamine reaction and by orcinol-HCl reaction, respectivley.

3) Nucleic acid solution used here contained 3.5 mg. of total protein per ml. The amount of total protein was determined by Greenberg's method (*9*) using phenol reagent according to Folin-Ciocalteu.

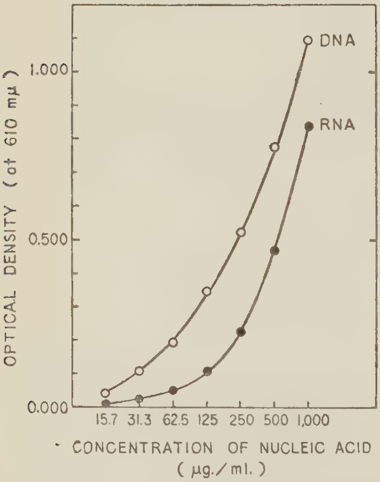

FIG. 1. Effect of varying concentrations of nucleic acids on the turbidity caused by precipitation of nucleic acids with a constant concentration of kanamycin. Final concentration of reaction system: kanamycin, 250 μg./ml. (in case of DNA) or 500 μg./ml. (in case of RNA); phosphate buffer, pH 6.0, *M*/60; DNA or RNA, varied.

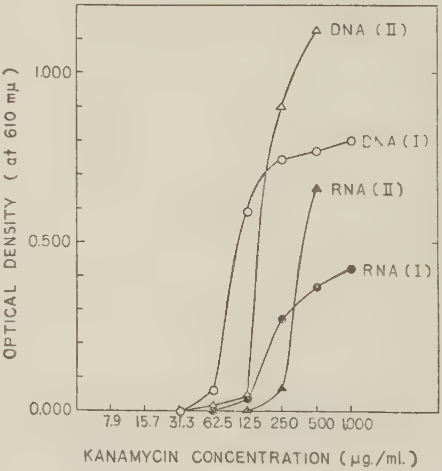

FIG. 2. Precipitation of nucleic acids by various concentrations of kanamycin—Relationship between nucleic acid concentrations and kanamycin concentrations. Final concentrations: (System I) DNA or RNA, 500 μg./ml.; phosphate buffer, pH 6.0, *M*/60; kanamycin, varied; (System II) DNA or RNA, 1,000 μg./ml.; phosphate buffer, pH 6.0, *M*/60; kanamycin, varied.

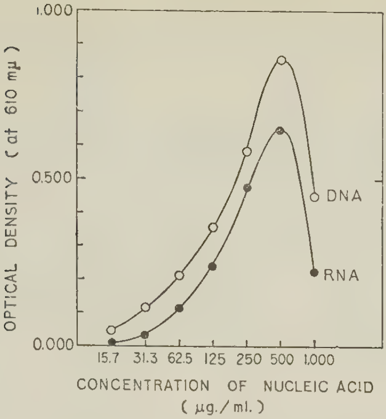

FIG. 3. Effect of varying concentrations of nucleic acids on the turbidity caused by precipitation of nucleic acids with a constant concentration of viomycin. Final concentrations of reaction system : viomycin, 500 μg./ml. ; phosphate buffer, pH 6.0, $M/60$; DNA or RNA, varied.

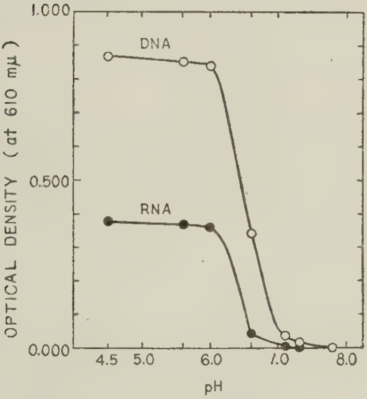

FIG. 4. Effect of hydrogen ions on the turbidity caused by precipitation of nucleic acids with kanamycin. Final concentrations : DNA or RNA, 500 μg./ml. ; kanamycin, 250 μg./ml. ; phosphate buffer, $M/60$ (pH varied).

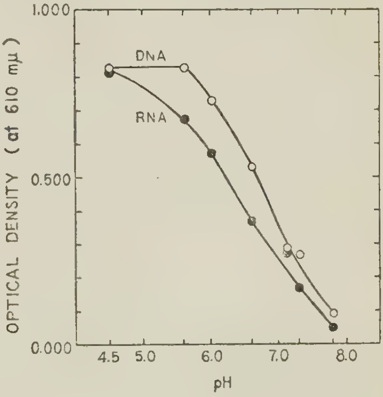

FIG. 5. Effect of hydrogen ions on the turbidity caused by precipitation of nucleic acids with viomycin. Final concentrations of reaction system : DNA or RNA, 500 μg./ml. ; viomycin, 500 μg/ml. ; phosphate buffer, $M/60$ (pH, varied).

Relationship between Drug Concentrations aud Nucleic Acid Concentrations—Precipitation of nucleic acids by kanamycin and viomycin was determined at various concentrations of nucleic acids and of the antibiotics. The results are shown in Figs. 1 to 3. Amounts of precipitates increased with increasing concentrations of nucleic acids in the presence of a constant concentration of the antibiotics (Figs. 1 and 3). They also increased with increasing concentrations of the antibiotics (Fig. 2). However, the presence of nucleic acids in excess decreased the amounts of precipitates (Figs. 2 and 3). It is noteworthy that the degree of precipitation is affected by interrelation between concentrations of the antibiotics and of the nucleic acids. If amounts of nucleic acids have been increased in reaction system, amounts of resultant precipitates increase at higher concentrations of the antibiotics but decrease at lower concentrations of the antibiotics. The degree of precipitation varies reversibly with varying relationship between concentrations of the antibiotics and of nucleic acids.

Effect of pH on the Precipitation of Nucleic Acids by Kanamycin and Viomycin—The degree of precipitation is significantly affected by the concentration of hydrogen ions. The amount of precipitates has increased with lowering the pH (Figs. 4 and 5). The effect of pH is more dramatic in precipitation of "Nucleic acids-Kanamycin" than in precipitation of "Nucleic acids-Viomycin" (Figs. 4 and 5).

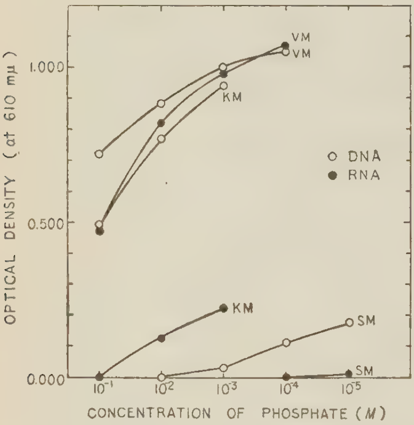

FIG. 6. Effect of varying concentrations of phosphate on the turbidity caused by precipitation of nucleic acids by streptomycin, kanamycin, and viomycin. Final concentrations of reaction system: DNA or RNA, 500 μg./ml.; kanamycin (KM), 250 μg./ml., viomycin (VM), 500 μg./ml, or streptomycin (SM), 500 μg./ml.; phosphate buffer, pH 6.0 (various molecular concentrations.

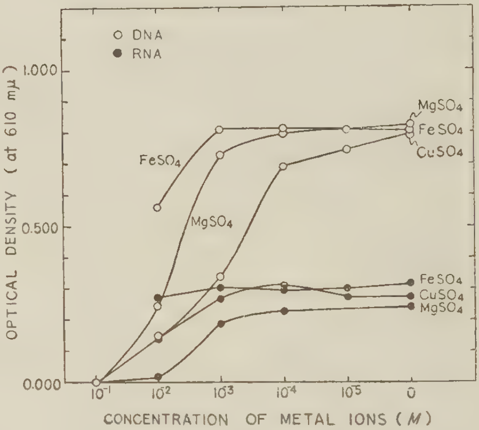

FIG. 7. Effect of certain cations on the turbidity caused by precipitation of nucleic acids by kanamycin. Final concentrations of reaction system: DNA or RNA, 500 μg./ml.; kanamycin, 250 μg./ml.; phosphate buffer, pH 6.0, $M/60$; $MgSO_4$-$7H_2O$, $CuSO_4$-$5H_2O$, or $FeSO_4$-$7H_2O$, varied.

Effect of Varying Concentrations of Phosphate on the Precipitation of Nucleic Acids by Kanamycin and Viomycin—The degree of precipitation is also significantly affected by the concentrations of phosphate. Amounts of precipitates have been determined at various molar concentrations of phosphate buffer. The amounts of precipitates have decreased with increasing concentrations of phosphate (Fig. 6).

Effect of Certain Cations on the Precipitation of Nucleic Acids by Kanamycin and Viomycin—Amounts of precipitates were determined at various concentrations of $MgSO_4$-$7H_2O$, $CuSO_4$-$7H_2O$, and $FeSO_4$-$7H_2O$. The addition of these

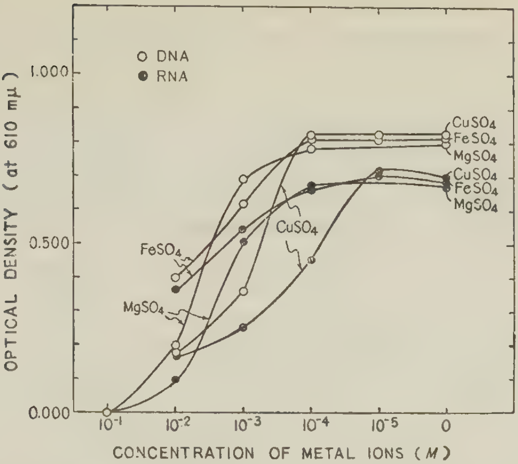

FIG. 8. Effect of certain cations on the turbidity caused by precipitation of nucleic acids by viomycin. Final concentrations of reaction system: DNA or RNA, 500 μg./ml.; viomycin, 500 μg./ml.; phosphate buffer, pH 6.0, M/60; $MgSO_4$-$7H_2O$, $CuSO_4$-$5H_2O$, or $FeSO_4$-$7H_2O$, varied.

cations (Mg^{++}, Cu^{++}, Fe^{++}) has more or less significantly affected the precipitation (Figs. 7 and 8), and amounts of precipitates have decreased by increasing concentrations of the cations. Generally speaking, the effect of Cu^{++} has appeared most significant, that of Mg^{++} moderately significant, and that of Fe^{++} less significant.

DISCUSSION

It has been demonstrated in the present study that kanamycin and viomycin precipitate DNA as well as RNA, and that their abilities to precipitate nucleic acids are reduced with increasing concentrations of phosphate and some metal cations and with lowering the pH. The properties of kanamycin and viomycin to precipitate nucleic acids are similar to those of streptomycin. However, it has been pointed out that these antibiotics precipitate nucleic acids much more significantly than streptomycin, and that they precipitate both DNA and RNA, whereas streptomycin precipitates practically DNA alone. This finding may suggest that these antibiotics might have an effect to disturb protein biosynthesis in bacteria sensitive to them.

SUMMARY

It has been demonstrated that kanamycin and viomycin precipitate desoxyribonucleate (DNA) and ribonucleate (RNA). Their ability to precipitate nucleic acids are much more significant than that of streptomycin, and they can precipitate both DNA and RNA.

The effect of kanamycin and viomycin on the precipitation of nucleic acids are affected by the interrelation between concentrations of nucleic acids and of the antibiotics. Lowering the pH and increasing concentrations of phosphate and some metal cations (Mg^{++}, Cu^{++}, Fe^{++}) decrease the degree of precipitation of nucleic acids by kanamycin and viomycin.

The authors wish to express their appreciation to Dr. K. Yagi, Nagoya University, for his kind adive. The authors are indebted to Dr. R. Katsunuma, the Obuso National Sanatorium, for his kind encouragement in the conduct of the present study.

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BIOCHEMICAL STUDIES ON THE FORMOSAN SNAKE VENOMS

IV. THE TOXICITY OF FORMOSAN COBRA VENOM AND ENZYME ACTIVITIES

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(Received for publication, February 9, 1959)

In the last thirty years extensive toxicological and biochemical studies on the six kinds of Formosan snake venoms have been made, constituting a characteristic problem in Taiwan. Despite the effort of many workers, the nature of snake venom and the mechanism of its toxicity upon animals are still clouded in mystery.

The development in the field of zone electrophoresis has been particularly rapid during the last few years, providing some very useful methods with many possible applications in enzymology as well as in protein chemistry. Snake venoms are complex mixtures of components, many of which are known to be proteins having enzymic activities. Recently Michl (*1*) and Ohsaka (*2*) have investigated by zone electrophoresis, the relation between enzymic activities and toxicity of the venoms of *Bothrops* and *Trimeresurus flavoviridis* respectively. However, there have been very few reports of electrophoresis, especially zone electrophoresis, studies concerning the Formosan snake venoms. Therefore, in the proceding study (*3*) zone electrophoresis of Formosan cobra venom and Hyappoda (*Agkistrodon acutus*) venom in a packed starch column were performed and the validity of the results obtained were established. It appeared that such a fractionation might be useful in a study of the relationship of enzyme activity to venom toxictiy.

In this study Formosan cobra venom was fractionated by zone electrophoresis into five protein fractions and the distribution of several enzyme activities together with toxicity in each fraction was surveyed. The primary thesis of these experiments is to elucidate the relationship of enzyme activity to toxicity in cobra venom. The results obtained indicate that the venom toxicity was separated from all of the enzyme activities studied.

EXPERIMENTAL

Materials and Methods—The venom used in this study was freshly collected Formosan cobra (*Naja naja atra*) venom, which was dried up with sulfuric acid in a vacuum desiccator in a cold room.

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* This paper was presented at the 51st Annual Meeting of the Formosan Medical Association, Taipei, November 22, 1958.

The disodium *p*-nitrophenyl phosphate used was a Daiichi product; adenosine triphosphate (ATP), a Wako product; and 5′-adenylic acid, a Nutritional Biochemical Corporation product. Ca [*bis*(*p*-nitrophenyl) phosphate]$_2$ synthesized by the method of De Garilhe and Laskowski (*4*) was kindly donated by Dr. T. Suzuki, Prof. the Pharmaceutical Institute, Medical Faculty, University of Kyoto.

Zone Electrophoresis: The zone electrophoresis was performed on a starch column with a vertical-type apparatus described by Naono (*5*). The experiment was carried out at about 8° in a veronal-HCl buffer, pH 8.6, ionic strength 0.1. 20 mg. of dried venom dissolved in 1 ml. of the veronal buffer was introduced at a height of 23 cm. in the starch stratum and the apparatus was equipped as described in Paper III (*3*). A current of 2.2 mA/cm.^{2} and 24.1 v./cm. was applied for 24 hours. After an electrophoresis had been concluded, the starch column was detached from the zone apparatus and the venom was eluted by allowing the same buffer solution to run slowly at a rate of approximately 0.42 ml. per minute through the column. 2 ml. fractions of the effluent were collected in a fraction collector and the amount of protein in each fraction was determined according to the method of Folin and Ciocalteu (*6*).

Measurement of the Enzyme Activities: Phosphomonoesterase, phosphodiesterase and 5′-nucleotidase activities were measured by the same method as described in Paper II (*7*). ATPase activity measurement followed exactly the method used for 5′-nucleotidase, except that the substrate, 5′-adenylic acid, was replaced by 0.02 *M* ATP. The enzyme unit was also defined in the same way. Phosphatidase A activity was measured by our improved method reported previously (*8*) and the enzyme activity was expressed by HD_{50} (50 per cent hemolytic dose).

Toxicity: Venom toxicity was measured by intraperitoneal injection of serial 2-fold dilution of each fraction into mice weighing from 12 g. to 16 g. in the amount of 0.2 ml. Four mice were injected per dilution. In order to diminish the untoward reaction due to the high concentration of veronal, dilutions were made with normal saline. The LD_{50} was calculated according to the 50 per cent endpoint method of Reed and Muench (*9*). The MLD and LD_{50} of the crude venom used were approximately 12 μg. and 8 μg. respectively.

RESULTS

The elution diagram of cobra venom subjected to zone electrophoresis in a packed starch column is presented in Figs. 1-4. The electrophoretic conditions are given in Fig. 1 and the arrow represents the place where crude venom was applied. Under this condition venom migrated toward the cathode and separated into five protein fractions. The validity of the elution diagram of starch column electrophoresis has been established by checking it with boundary electrophoresis using Spinco H (*3*). The protein recovery was 102.0 per cent.

The distribution of enzyme activities in each fraction were surveyed with the results shown in Figs. 1, 2 and 3. Phosphodiesterase and phosphomonoesterase activities spread over fractions No. 26 to 48 (Fig. 1). However, more than 90 per cent of the recovered phosphodiesterase activity was concentrated in fractions No. 28 to 38 and constituted a high peak just between the two protein fractions, 4th and 5th protein fraction, where the protein content was lowest. There is still another small peak around the starting point, when expressed by specific activity. The recovery of phosphodiesterase and phosphomonoesterase activities were 65.8 per cent and 77.5 per cent respectively. Whereas adenosine triphosphatase (ATPase) activity spread over fractions No. 20 to 45 and formed one major peak and three small peaks, by specific activity, 5′-nucleotidase activity was distributed in rather narrow range and constituted a high peak at 4th protein fraction (Fig. 2). 5′-Nucleotidase and ATPase activities recovered were 70.2 per cent and 60.2 per cent respectively. The distribution of phosphatidase A activity was surveyed by our improved method with the result shown in Fig. 3. The peak of phosphatidase A activity coincided well with the 5th protein fraction and revealed that only electrophoretically single fraction is involved in cobra venom. The phosphatidase A activity recovery was 245.2 per cent. One possible explanation for the high percentage of recovery is that there may be some factor(s) inhibiting phosphatidase A activity in crude venom, which are separated or removed by zone electrophoresis.

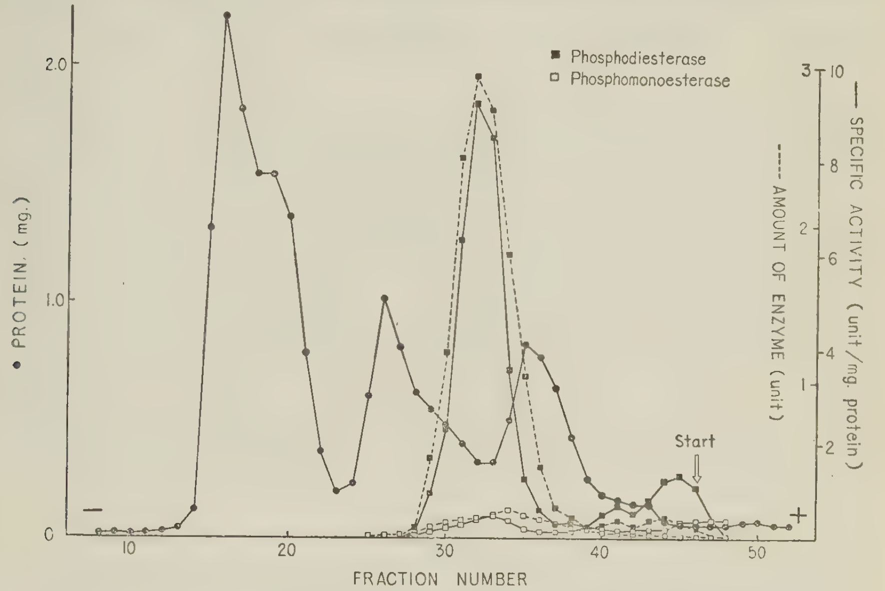

FIG. 1. Zone electrophoretic pattern of Formosan cobra venom protein together with phosphodiesterase and phosphomonoesterase activities.

20 mg. of dried venom in veronal-HCl buffer, pH 8.6, ionic strength 0.1, was subjected to electrophoresis in a cloumn 3.5×40 cm. packed with starch. The experiment was performed in a cold room (8° as measured). A current of 21.2 mA. (2.2 mA/cm^2., 24.1 v./cm.) was applied for 24 hours. The protein concentration of the fractions was determined by Folin's method (*6*) and protein recovery was 102.0 per cent. Distribution of protein —•—; phosphodiesterase activities – –■– – ; phosphomonoesterase activities - -□- - ; and specific activities per mg. of protein of the enzymes; phosphodiesterase —■— ; phosphomonoesterase —□—.

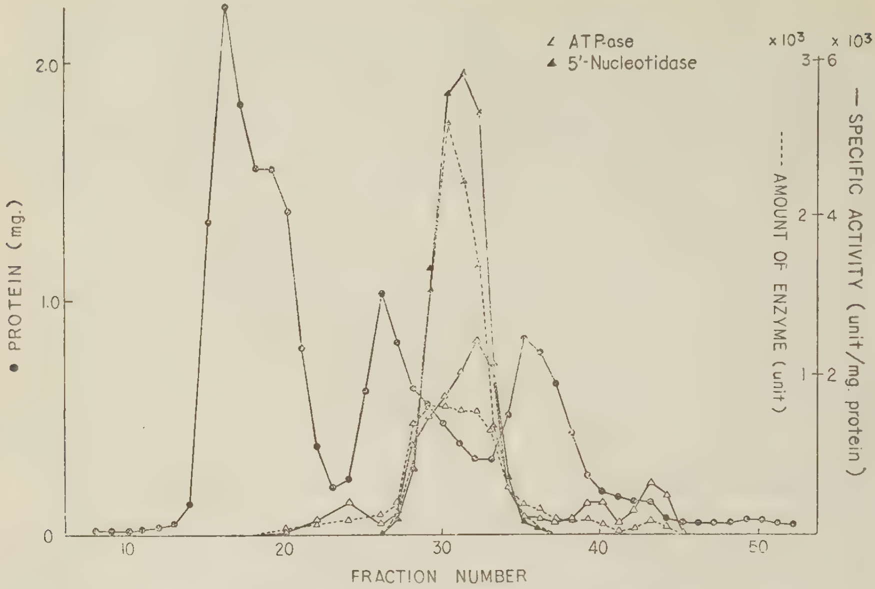

FIG. 2. Distribution of 5′-nucleotidase (- -▲- -) and ATPase (- -△- -) activities and the specific activities per mg. of protein of the 5′-nucleotidase (—▲—) and ATPase (—△—) in the same electrophoretic run as in Fig. 1.

TABLE I

Specific Activities of Enzymes and of Toxicities per Unit Amount of Protein in Crude Cobra Venom and in Eluates After Zone Electrophoresis

Enzymes	Specific activity (*unit per mg. protein*)		Specific activity increased (*fold*)
	Crude venom1)	Eluates	
Phosphodiesterase	1.078	9.25 (32)2)	8.6
Phosphomonoesterase	0.101	0.49 (33)	4.9
5′-nucleotidase	720	5817 (31)	8.1
ATPase	580	2459 (32)	4.2
Phoshatidase A (HD$_{50}$)	11,007	182,225 (35)	16.5
Toxicity (LD$_{50}$)	125	316.5 (27)	2.5

1) The data were cited from another series of our work (10).
2) The figures in parentheses denote the fraction number.

The distribution of toxic principle(s) in the venom was represented both by MLD and LD$_{50}$ in Fig. 4. The recoveries calculated from the starting material of toxicity were 51.8 per cent for MLD and 63.2 per cent for LD_{50}. As Fig. 4 shows, although there are some toxic principles in 1st and 2nd protein fractions which make up 25 per cent of recovered toxicity, the majority of the toxicity, however, which represent approximately 75 per cent of the recovered toxicity, was concentrated in 3rd and 4th protein fractions. It is more clearly expressed by specific activity of toxicity per unit amount of protein that besides one major toxic peak in 3rd and 4th protein fractions there are two minor toxic peaks in 1st and 2nd protein fractions. Therefore, it seems to us that at least three toxic principles may be involved in the crude cobra venom.

It is apparent that all of the enzymes tested were separated from toxicity; phosphatidase A was separated from toxicity particularly distinctly. Furthermore, as may be seen from Table I, simply by means of starch column electrophoresis the specific activity of toxicity and many enzymes were increased several fold to even more than ten times. The method for the separation and purification of snake venoms is felt to represent an improvement over the precipitation method using salts or some organic solvents, since this separation is such a gentle procedure, involving only migration in a medium of approximately constant composition.

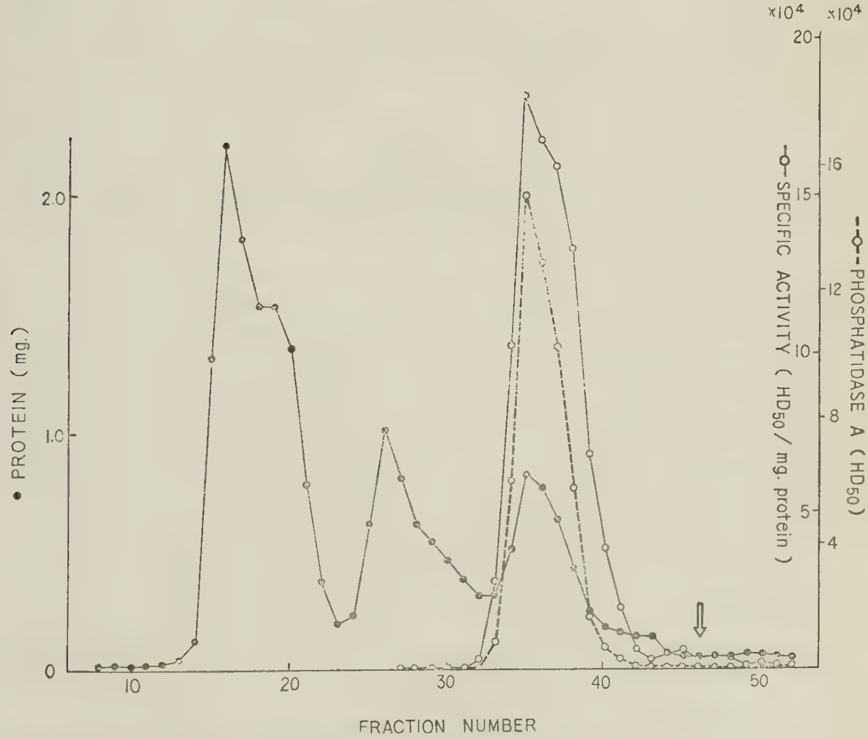

FIG. 3. Distribution of phosphatidase A activities (- -○- -) and the specific activities (—○—) per mg. of protein in the same electrophoretic run as in Fig. 1.

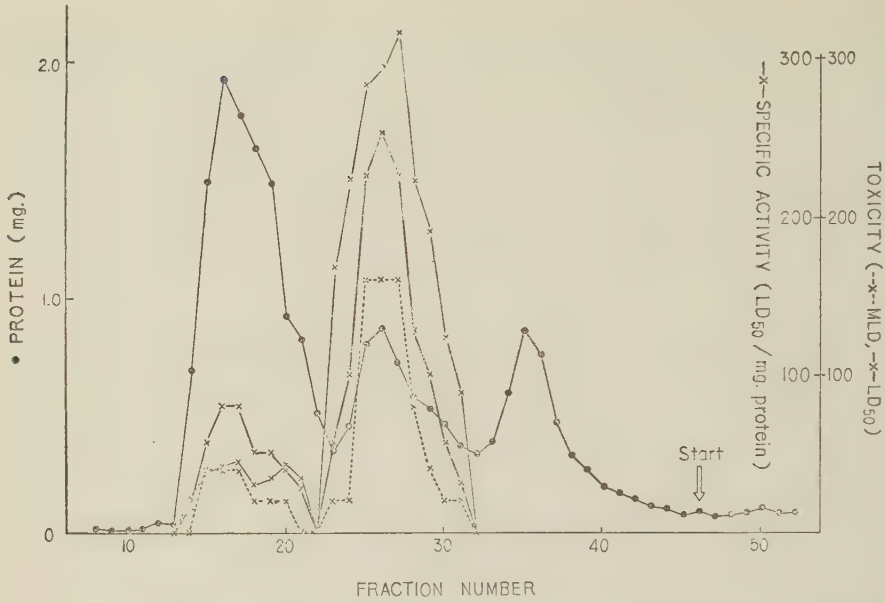

Fig. 4. Distribution of toxicities; MLD (--×--) and LD_{50} (—×—) and the specific activities per mg. of protein of the LD_{50} —×— in another electrophoretic run in which the experimental conditions were kept practically the same as in Fig. 1.

DISCUSSION

While Taborda *et al.* (*10*) suggested that the average phosphodiesterase content in venoms of various species run parallel to their toxicity, the phos-

TABLE II

*Phosphodiesterases and Toxicities of Formosan Snake Venoms**

Venoms	Phosphodiesterase	Toxicity (LD_{50})
	(*unit per mg. of dried venom*)	
Naja naja atra	1.078	125
Bungarus multicinctus	0.022	2000
Vipera russellii formosensis	3.955	200
Trimeresurus macrosquamatus	3.008	36
Trimeresurus gramineus	3.116	76
Agkistrodon acutus	1.382	18

* The data were cited from another series of our work (*10*).

phodiesterase activity in cobra venom was separated from the toxicity in good accordance with Ohsaka's finding (*2*). Furthermore, comparison of the phosphodiesterase content in six kinds of Formosan snake venoms with their toxicities revealed that no relationship exists between the two (Table II).

Zeller (*12*) believed that ophio-ATPase together with phosphomonoesterase and 5′-nucleotidase are probably responsible for the production of fatal shock by exhausting supplies of readily available energy in the form of ATP and creatinephosphoric acid. However, as it was shown in the preceding section, these enzymes are all separated from the venom toxicity.

In agreement with Slotta's finding (*13*), we found that at pH 8.6 the cobra venom gave electrophoretically only one single fraction of phosphatidase A, while Grassmann and Hannig (*14*) and Ohsaka (*2*) have observed two hemolytic fractions from their venoms with paper or zone electrophoresis. Phosphatidase A activity was separated from venom toxicity very distinctly, and even intraperitoneal injection of 7535 units (HD_{50}) of the enzyms (fraction No. 35) did not cause any toxic effect on mice at all. Therefore, it seems likely that phosphatidase A has nothing to do with the toxic nature of the venom. The relatively nontoxic crystalline hemolytic "lecithinase" preparation had been separated from the neurotoxic components of Indian cobra venom by De (*15*). Recently the separation of phosphatidase A from venom toxicity by zone electrophoresis or by the chromatographic procedure was also announced by Bussard and Côté (*16*), Neumann and Habermann (*17*), and Ohsaka (*2*).

It would be expected that if a given enzyme action of snake venom causes its toxicity, then the distribution curve of the enzyme activity should coincide with the peak of toxicity. Although it has been reported by several authors that enzymic activities of cobra venom seem to be connected with the toxic fractions of cobra venom were separated from the enzyme activities tested. Since, proteinase activity in cobra venom is so feeble (0.65) $[\mathrm{PU}]^{\mathrm{Cas.\ FR.\ A.}}_{\mu\mathrm{g.\ tyr.}}$ per mg. of dried venom (*10*), it is impossible to determine the enzyme activity in each fraction. It appears that none of the enzyme activities studied offers an explanation for the toxic action of cobra venom. During preparation of this paper Radomski and Deichmann (*18*) reported that they separated *Naja flava* venom into two fractions by ethanol, and demonstrated that the more toxic fraction was much less potent with respect to phosphatidase A, adenosine triphosphatase, diphosphopyridinenucleotidase, and ribonuclease activities. They concluded that none of these enzyme activities is of prime importance toxicologically. However, whether the venom toxicity has absolutely nothing to do with any enzyme activities or not remains to be ascertained. Further studies on the toxic nature of snake venoms are now being continued.

SUMMARY

1. Formosan cobra (*Naja naja atra*) venom was fractionated by zone electrophoresis in a packed starch column into five protein fractions.

2. The distribution of several enzyme activities and toxicity in each fraction were surveyed. All of the enzymes studied were separated from toxicity, above all phosphatidase A was separated from toxicity very distinctly.

3. At pH 8.6 cobra venom gave electrophoretically only one single fraction of phosphatidase A.

4. It was shown that at least three toxic principles were present in this venom and also proved that they have no relation with the enzymes, phosphodiesterase, phosphomonoesterase, 5′-nucleotidase, ATPase, proteinase, and phosphatidase A.

The authors are gratefully indebted to Prof. R. Hirohata, chief of the Laboratory of Protein Chemistry, Yamaguchi Medical School, Ube, for his continued interest and encouragement during the course of this study.

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BIOCHEMICAL STUDIES ON THE FORMOSAN SNAKE VENOMS

V. THE TOXICITY OF HYAPPODA (AGKISTRODON ACUTUS) VENOM AND ENZYME ACTIVITIES*

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(Received for publication, February 9, 1959)

It was demonstrated by zone electrophoresis study (*1*) that the toxicity of Formosan cobra (Elapidae) venom has no relation with the enzymes, phosphodiesterase phosphomonoesterase, 5′-nucleotidase, ATPase, proteinase, and phosphatidase A. We are also interested in the problem of what relationship exists between toxicity and enzyme activities in the venoms of many different species. The electrophoretic properties of Hyappoda, *Agkistrodon acutus* (*Crotalidae*) venom are proved to be quite distinct from cobra venom and the validity of the electrophoretic pattern obtained with the venom in a packed starch column was established in the previous study (*2*). Therefore, in the present study the Hyappoda venom was fractionated by zone electrophoresis and the distribution of toxicity and of several enzyme activities in each fraction was surveyed. The results obtained indicate that the venom toxicity was separated from phosphodiesterase and phosphatidase A but the first toxic peak coincided with the first peak of proteinase activity and the second toxic peak, with 5′-nucleotidase and ATPase activities.

EXPERIMENTAL

Materials and Methods—The venom used in this study was freshly collected Hyappoda (*Agkistrodon acutus*) venom which was dried up with sulfuric acid. Substrates used were the same as described in Paper IV (*1*). Zone electrophoresis, measurement of the enzyme activities and of the toxicity followed exactly the method used for the study of cobra venom (*1*). The MLD and LD_{50} of the crude venom used were approximately 80 μg. and 56 μg. respectively.

RESULTS

The elution diagram of Hyappoda venom subjected to zone electrophoresis is shown in Figs. 1∼4. The electrophoretic conditions are given in Fig. 1 and the arrow represents the place where crude venom was applied, *i.e.*, at a height of 23.5 cm. in the starch stratum. Under this condition venom migrated toward the anode and separated into one main protein fraction, representing

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* This paper was presented at the 51st Annual Meeting of the Formosan Medical Association, Taipei, November 22, 1958.

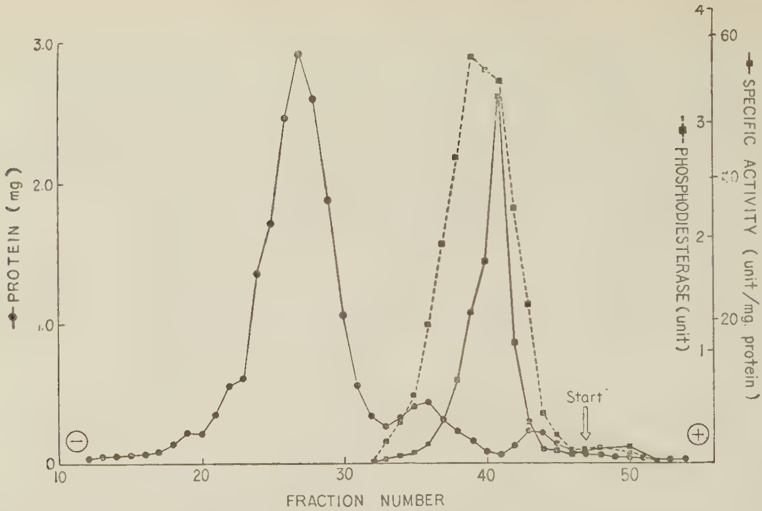

FIG. 1. Zone electrophoretic pattern of Hyappoda venom, protein together with phosphodiesterase activities.

20 mg. of dried venom in veronal-HCl buffer, pH 8.6, ionic strength 0.1, was subjected to electrophoresis in a column 3.5×40 cm. packed with starch. The experiment was performed in a cold room (8° as measured). A current of 21.2 mA. (2.2 mA/cm^2., 24.7 v./cm.) was applied for 24 hours. The protein concentration of the fractions was determined by Folin's method (*3*) and protein recovery was 103.8 per cent. Distribution of protein —•—; phosphodiesterase activities --■--; the specific activities per mg. protein of phosphodiesterase —▨—.

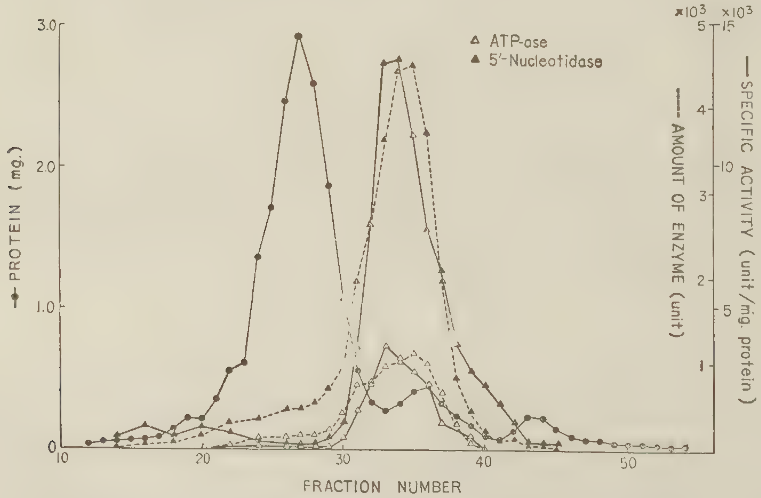

FIG. 2. Distribution of 5′-nucleotidase (--▲--) and ATPase (--△--) activities and the specific activities per mg. of protein of the 5′nucleotidase (—▲—) and ATPase (—△—) in the same electrophoretic run as in Fig. 1.

83.5 per cent of the recovered protein, and two minor protein fraction making up 16.5 per cent. Electrophoretic separation of Formosan snake venoms at pH 8.6 with a sodium diethylbabiturate buffer, ionic strength 0.1, revealed that except for Hyappoda venom which migrated toward the anode, the other venoms: *Naja naja atra* (cobra), *Bungarus multicinctus*, *Vipera russellii formosensis*, *Trimeresurus macrosquamatus*, and *Trimeresurus gramineus* venoms, all migrated towards the cathode.

The distribution of enzyme activities in each fraction was surveyed and the results obtained are presented in Figs. 1, 2, 3 and 4.

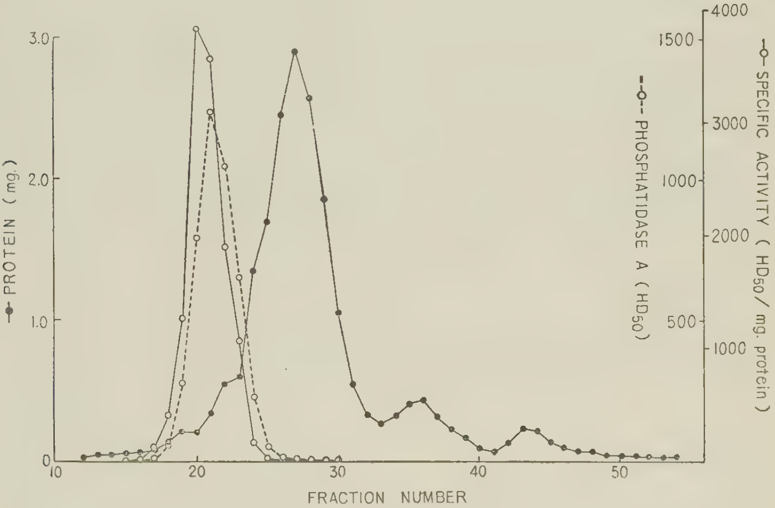

FIG. 3. Distribution of phoshatidase A activities (- -○- -) and the specific activities (—○—) per mg. of protein in the same electrophoretic run as in Fig. 1.

As Fig. 1 shows, phosphodiesterase activity was distributed in a rather narrow range and constituted a high peak just between the two minor protein fractions. While Formosan cobra venom possesses a small peak of phosphodiesterase activity in addition to the main peak, Hyappoda venom exhibits only one electrophoretically single fraction of phosphodiesterase. The recovery of phosphodiesterase activity was 80.6 per cent and the specific activity in fraction No. 41 increased from 1.382 to 51.85 units per mg. of protein, *i.e.*, a 37.5 fold increase from crude venom (Table I). Since, phosphomonoesterase activity in Hyappoda venom is feebly appreciated, 0.013 unit per mg. of dried venom, it is impossible to determine their activities in each fraction. 5′-Nucleotidase and ATPase activities spread over almost all of the fractions. However, more than 80 per cent of the recovered enzyme activities were concentrated around the 1st minor protein fraction and constituted high peaks (Fig. 2). The recoveries of 5′-nucleotidase and ATPase activities were 118.6 per cent and 44.5 per cent respectively. It is clearly demonstrated in

TABLE I

Specific Activities of Enzymes and of Toxicities per Unit Amount of Protein in Crude Hyappoda Venom and in Eluates after Zone Electrophoresis

Enzymes	Specific activity (*unit per mg. protein*)		Specific activity increased (*fold*)
	Crude venom[1]	Eluates	
Phosphodiesterase	1.382	51.85 (41)[2]	37.5
5′-Nucleotidase	1211	13759 (34)	11.4
ATPase	909	3678 (33)	4.0
Phosphatidase A (HD_{50})	331	3850 (20)	11.6
Proteinase [PU] Cas. FR. A. / μg. try.	71.64	485.4 (46)	6.8
Toxicity (LD_{50})	18	24.3 (33)	1.4

1) The data were cited from another series of our work (*4*).

2) The figures in parentheses denote the fraction number.

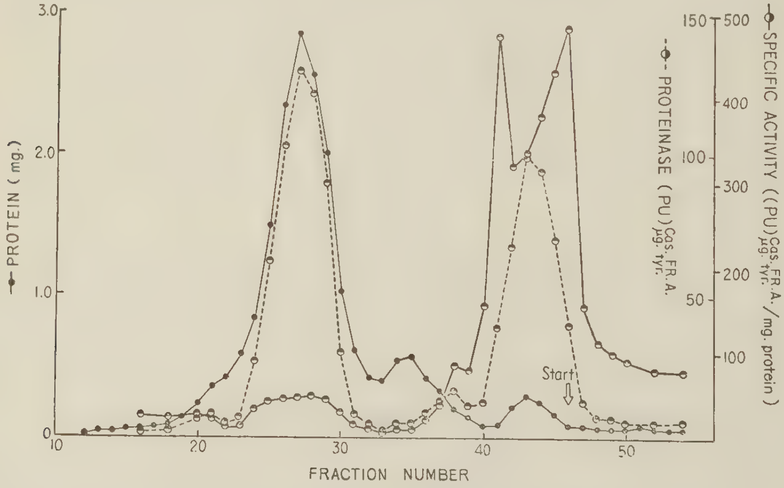

FIG. 4. Distribution of proteinase activities (--◓--) and the specific activities (—◓—) per mg. of protein in another electrophoretic run in which the experimental conditions were kept practically the same as in Fig. 1.

Fig. 3, that only one electrophoretically single fraction of phosphatidase A activity was obtained towards the beginning of the main protein fraction. This finding has a strong resemblance to the result obtained with Formosan cobra venom (*1*) and Slotta's observation (*5*). The phosphatidase A activity recovery was 66.6 per cent. The data of proteinase activities in each fraction cited from another series of our work (*6*) are presented in Fig. 4. Two peaks are in existence coincidentally with the main and the 2nd minor protein fractions. Furthermore, the peaks followed exactly the same pattern of protein. Though the first peak of proteinase activity was some what higher than the second one, it became a low flat peak by specific activity. However, the second proteinase peak exhibited an even higher peak.

The distribution of toxic principle(s) in the venom was represented both by MLD and LD_{50} in Fig. 5 with recoveries of 30.1 per cent and 63.2 per cent respectively. Because of the low recovery of MLD, LD_{50} was considered to be a superior estimate for the expression of venom toxicity. Two toxic fractions were obtained; the major one which coincides with the main protein fraction amounts to 86.2 per cent of the recovered toxicity (LD_{50}) and another small toxic fraction which gives only 138 per cent of recovered toxicity is located at the 1st minor protein fraction. However, the specific activity of these two toxic peaks were almost the same. It is therefore suggested that at least two toxic principles are present in the Hyappoda venom.

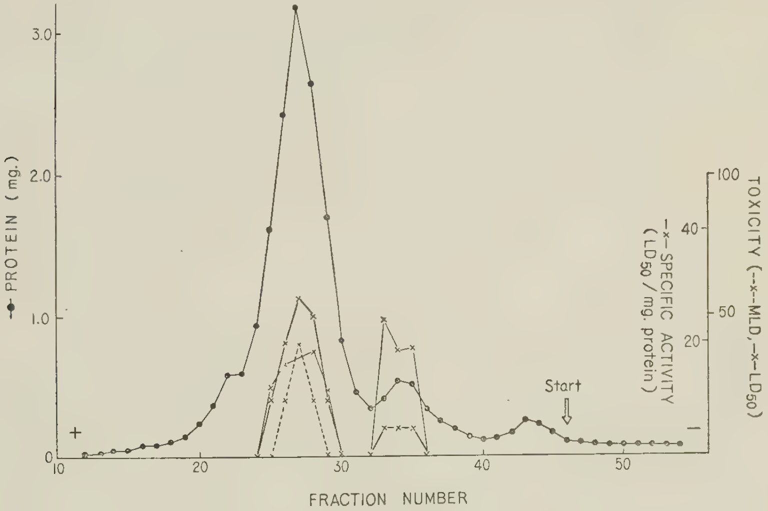

FIG. 5. Distribution of toxicities; MLD (- - × - -) and LD_{50} (—×—) and the specific activities per mg. of protein of the LD_{50} (—×—) in another electrophoretic run in which the experimental conditions were kept practically the same as in Fig. 1.

Table I shows the increment in specific activities of enzymes and of toxicities by zone electrophoresis.

DISCUSSION

Electrophoretic separation of Formosan snake venoms at pH 8.6 with a sodium diethylbarbiturate buffer, ionic strength 0.1, revealed that only Hyappoda venom migrated toward the anode. At pH 7.6 with a phosphate buffer, ionic strength 0.1, the venom was still migrating towards the anode (*2*), indicating that the isoelectric points of the venom proteins are below 7.6.

In this study, phosphodiesterase and phosphatidase A activities were also separated from venom toxicity distinctly, proving that no relationship exists between these enzyme activities and venom toxicity. Furthermore, phosphodiesterase activity was separated from 5′-nucleotidase and ATPase activities and the specific activity increased 37.5 fold by zone electrophoresis, in addition to this phosphomonoesterase activity in original Hyappoda venom was feebly enough. Therefore, the phosphodiesterase preparation obtained in this study may provide a valuable tool for the study of nucleic acid chemistry.

The peaks of 5′-nucleotidase and ATPase activities coincide with the second toxic peak. This finding tends to confirm Zeller's suggestion that both enzymes are probably responsible for the production of fatal shock (*7*). However, comparison of the 5′nucleotidase and ATPase content in six kinds of Formosan snake venoms with their toxicities revealed that no relationship exists between each other (Table II).

TABLE II

*5′-Nucleotidase, ATPase, and Toxicity of Formosan Snake Venoms**

Venoms	Enzymes		Toxicity (LD_{50})
	5′-Nucleotidase	ATPase	
	(unit per mg. of dried venom)		
Naja naja atra	720	580	125
Bungarus multicinctus	6	12	2000
Vipera russellii formosensis	270	227	200
Trimeresurus macrosquamatus	2140	1344	36
Trimeresurus gramineus	2320	1620	76
Agkistrodon acutus	1211	909	18

* The data were cited from another series of our work (*4*).

Although the first peak of proteinase activity coincides with the first toxic peak, the second proteinase fraction did not cause any toxic effect on mice at all. Therefore, it seems doubtful that the proteinase activities are connected with the toxic action of the first toxic peak. However, at present we dare not decide whether venom toxicity has absolutely nothing to do with the enzyme activities and the studies are now being continued.

SUMMARY

1. Hyappoda (*Agkistrodon acutus*) venom was fractionated by zone electro-

phoresis in a packed starch column and the distribution of several enzyme activities and toxicity in each fraction were surveyed.

2. Two toxic principles were proved to be involved in the venom.

3. Phosphodiesterase and phosphatidase A activities were obtained as an electrophoretically single fraction and both were separated from venom toxicity distinctly.

4. The peaks of 5′nucleotidase and ATPase activities coincide with the second toxic peak and the first peak of proteinase activity coincides with the first toxic peak. A possible explanation for the observed results was discussed.

The authors ars gratefully indebted to Prof. R. Hirohata, chief of the Laboratory of Protein Chemistry, Yamaguchi Medical School, Ube, for his continued interest and encouragement during the course of this study.

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STUDIES ON ACETIC ACID-BACTERIA

I. BIOCHEMICAL STUDIES ON ETHANOL OXIDATION*

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(Received for publication, February 11, 1959)

Vinegar production is in principle based upon a fermentation by acetic acid-bacteria growing in a medium containing ethanol. The quality of the vinegar produced will naturally be dependent mainly on the fermentative properties of bacteria, the culture medium and conditions applied. In commercial production of vinegar, investigations have mostly been directed to the improvement of the quality of vinegar. However much remains to be known how to regulate the oxidative fermentation to make vinegar uniform. Oxidative fermentation of ethanol by acetic acid-bacteria has been studied by many investigators. Tamiya and Tanaka (*1*) and Tanaka (*2*, *3*) reported that ethanol oxidation by a strain of *Acetobacter* was inhibited by carbon monoxide, and that this inhibition was restored by light-irradiation. They also found that the fermentation was inhibited by cyanide.

This paper reports biochemical studies on the oxidative fermentation of ethanol by *Acetobacter sp.* with special reference to the accumulation of much acetic acid in the system as a metabolic intermediate of ethanol.

MATERIALS AND METHODS

The strain of *Acetobacter* was that which is chiefly used in the vinegar industrial factory of Sasada Co., Ltd., Higashi-Nada, Kobe. The bacteria grew, forming a thin white film, and did neither utilize inorganic nitrogen nor form any pigment in the presence of glucose as the carbon source. This microorganism showed properties very similar but not the same to *Acetobacter suboxydans*, for it could oxidize acetic acid though much slower than ethanol.

The standard medium was composed of 5 g. of glucose, 10 g. of glycerol, 2 g. of peptone, 2 g. of yeast extract, a trace of magnesium sulfate and 20 ml. of 95 per cent ethanol in 1 liter of water (pH 3.5). In some cases, 20 ml. of acetic acid was added to the standard medium. The bacteria were grown aerobically for 4 days at 30°.

The grown cells were harvested and washed several times with distilled water. They were finely ground in a mortar and suspended in distilled water. The resulting suspension was used as the cell suspension.

The washed cells were ground with 5 volumes of sea sands and several volumes of dry ice for approximately one hour and the resulting frozen powder was then thawed in a water bath at 30°. This procedure was repeated. The resulting cellular paste was suspended in 20 volumes of 0.02 *M* phosphate buffer of pH 7.0. After standing overnight in a refrigerator, the suspension was centrifuged at 10,000×*g* for 30 minutes. After recentrifugation, the supernatant was used as cell-free extract (A). The cells ground with sea sand in the absence of dry ice gave a cell-free extract with a lower enzyme activity. In some cases, the washed cells were ground with 5 volumes of alumina powder in an ice-cold mortar for approximately one hour and the resulting cellular paste was extracted with 20 volumes of phosphate buffer in the same way as that described above for the cell-free extract (A). This extract was used as cell-free extract (B).

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* This work was done in the laboratory of Prof. K. Okunuki, Department of Biology, Faculty of Science, University of Osaka, Osaka.

The acid concentration was determined by titration with 0.002 *N* NaOH in the presence of phenolphthaleine.

The optical density was measured in a Shimadzu photoelectric spectrophotometer, type QB-50, at room temperature. The differential extinction of the cell suspension was measured with the aid of an opal glass according to the method of Calvin and Shibata (*4*).

RESULTS

Effect of Various Inhibitors on Ethanol Oxidation—The cell suspension rapidly oxidized ethanol, and acetaldehyde. Ethanol oxidation by the cell suspension was strongly inhibited by a low concentration of potassium cyanide, as shown in Table I. This inhibition occurred even in the presence of methylene blue.

TABLE I

Effect of Cyanide on Ethanol Cxidation by the Cell Suspension

Reaction mixture: 1 ml. of 0.2 *M* phosphate buffer of pH 7.0, 0.2 ml. of potassium cyanide, 0.2 ml. of 0.01 *M* methylene blue, 0.2 ml. of 0.1 *M* ethanol, and 0.5 ml. of cell suspension (approximately 6 mg. dry weight/ml.). Total volume adjusted to 2.4 ml. with distilled water. Center well of Warburg vessels contained 0.2 ml. of 20 per cent potassium hydroxide. Gas phase, air. Temperature, 30°. Ethanol-oxidizing activity expressed as amount of oxygen consumed during the initial 30 minutes.

Final concentration (*M*)		Ethanol-oxidizing activity ($\mu l.$)	Per cent inhibition
KCN	Methylene blue		
—	—	119	0
7.7×10^{-4}	—	20	84
7.7×10^{-4}	+	18	85
1.5×10^{-3}	—	12	90
3.0×10^{-3}	—	11	92

Ethanol was rapidly oxidized not only by the cell suspension but also by the cell-free system irrespective of the extraction method used. Acetaldehyde was oxidized further only by the cell-free extract (B), and not by the cell-free extract (A). Neither of the extracts, however, oxidized acetic acid at all. The ethanol-oxidizing activity of the cell-free extracts was associated with a particulate fraction precipitated by high speed-centrifugation (30,000 × g, 30 minutes) and the activity of the precipitate was independent of added coenzymes. Ethanol oxidation by cell-free extracts was also strongly inhibited by cyanide, and as in the case of the cell suspension, this inhibition was not restored by the addition of methylene blue, as shown in Fig. 1.

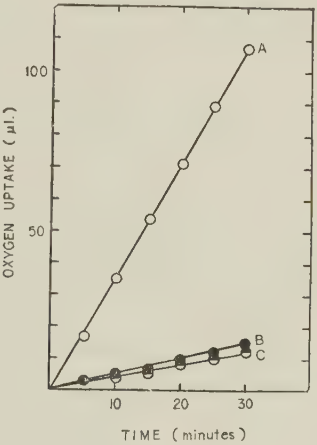


FIG. 1. Influence of cyanide on ethanol oxidation of cell-free extract. Experimental conditions were the same as for Table I except that cell-free extract (A) and 10^{-4} M potassium cyanide were used instead of cell suspension and various concentrations of potassium cyanide, respectively, and no potassium hydroxide was put into center well. Cell-free extract (B) gave the same results as cell-free extract (A). Curve A, ethanol; curve B, ethanol and cyanide; curve C, ethanol, cyanide and methylene blue.

Ethanol oxidation by the cell suspension was strongly inhibited by carbon monoxide, as shown in Table II. The activity could be restored to a large extent by light irradiation. A similar phenomenon was also observed in ethanol oxidation by cell-free extracts.

2,3-Mercaptopropanol (BAL), *p*-chloromercuribenzoate (PCMB) and urethane strongly inhibited ethanol oxidation by both the cell suspension and cell-free extracts, as shown in Table III. These inhibitors all inhibited ethanol oxidation by the cell-free extract more strongly than that by the cell suspension.

Interrelationship of the Oxidation of Ethanol and Acetic Acid—The strain of *Acetobacter* used in this experiment could grow as well in the medium supplemented with up to 7 per cent ethanol in final concentration, as in the standard medium, but bacterial growth was inhibited on addition of more than 4 per cent of acetic acid to the standard medium. Cells grown in the standard

TABLE II

Effect of Carbon Monoxide on Ethanol Oxidation of Cell Suspension

Experimental conditions as for Table I except that the gas phase was 5 per cent oxygen and 95 per cent nitrogen or carbon monoxide and the reactions were carried out at 30° and at 13° in the light (60 w. × 10 cm.) and in the dark.

	Per cent inhibition		Per cent reactivation
	In dark	In light	
Experiment I (at 30°)	96	79	8
	92	82	11
Experiment II (at 13°)	69	17	76
	68	31	55

TABLE III

Effect of Various Inhibitors on Ethanol Oxidation of Cell Suspension and of Cell-free Extract (B)

Experimental conditions as for Table I except for the addition of various inhibitors.

	Inhibitor	Final concentration	Per cent inhibition
Cell suspension	BAL	$6.7 \times 10^{-3}\,M$	80
	PCMB	$7.7 \times 10^{-5}\,M$	9
	Urethane	20%	31
Cell-free extract (B)	BAL	$6.7 \times 10^{-3}\,M$	93
	PCMB	$7.7 \times 10^{-6}\,M$	14
		$3.9 \times 10^{-5}\,M$	65
		$7.7 \times 10^{-5}\,M$	79
	Urethane	20%	58

medium could oxidize acetic acid very slowly compared with the oxidation of ethanol. However, cells grown in the medium containing acetic acid could oxidize it more rapidly than cells grown in the standard medium. Moreover, ethanol oxidation of cells was hardly influenced by whether they were cultured in the presence or absence of ethanol.

Suspensions of cells grown in the standard medium oxidized ethanol and acetic acid, as shown in Fig. 2. The acid formation rapidly increased during the first 35 minutes, but ceased when the ethanol added had apparently been exhausted. Then ethanol oxidation proceeded at the same speed as acetic acid-oxidation, and the acid concentration decreased due to acetic acid-oxidation. The maximal amount of acid produced during ethanol oxidation was almost equimolar to the ethanol added.

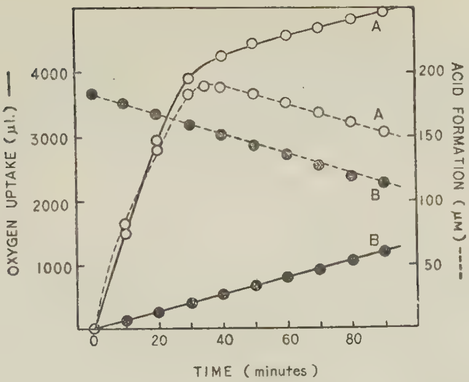


FIG. 2. Oxidation of ethanol and acetic acid, and formation of acetic acid during oxidation. For assay of the acid formed during the oxidations of ethanol and acetic acid, reactions were carried out in 500 ml.-flasks with 50 ml. of cell suspension (approximately 3 mg. dry weight per ml.) and 10 ml. of 0.02 *M* ethanol or acetic acid. For measurement of oxygen uptake the reaction was carried out in a Warburg vessel with 0.2 ml. of 20 per cent potassium hydroxide in the center well. Temperature, 30°. Full lines, oxygen uptake; broken lines, concentration of acid. Curve A, ethanol oxidation; curve B, acetic acid oxidation.

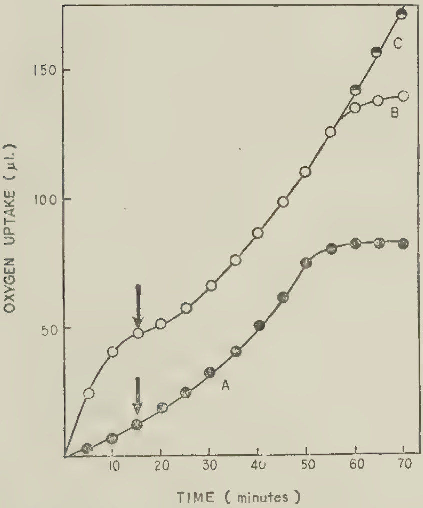


FIG. 3. Oxidation of ethanol and acetic acid by cell suspension. Experimental conditions as for Table I except that cells were grown in acetic acid-containing medium and 0.02 *M* ethanol or sodium acetate was used as substrate. Curve A, acetate; curve B, ethanol; curve C, acetate and ethanol. At the time showed by the arrow, evolution of carbon dioxide was measured after addition of sulfuric acid.

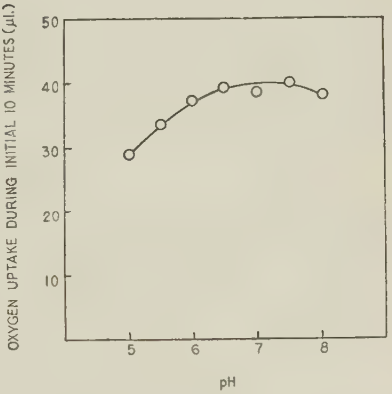


FIG. 4. Effect of pH on ethanol oxidation by cell suspension.

Experimental conditions as for Table I, except for use of phosphate buffer of various pH's. Ethanol-oxidizing activities measured by oxygen consumption during the initial 10 minutes.

Cells grown in the medium containing acetic acid were able to oxidize the acid much more rapidly than cells grown in the standard medium. The time course of ethanol oxidation was rather complicated, as shown in Fig. 3. At the time shown by the arrow in Fig. 3, the R.Q. values were 1.03, 0.02 and 0.02 when acetic acid only, ethanol only, and acetic acid plus ethanol respectively were added as substrates. After this time, the R. Q. for acetic acid-oxidation hardly changed until the substrate had been exhausted while the R. Q. for ethanol, and for acetic acid with ethanol rose to the value for acetic acid.

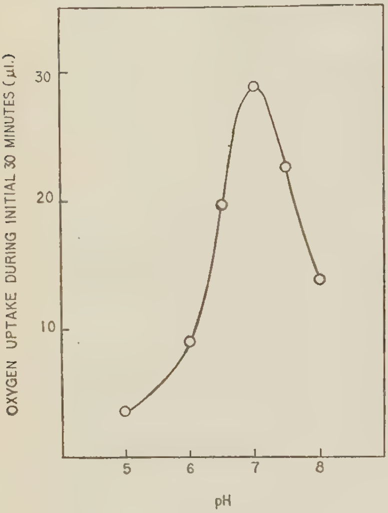


FIG. 5. Effect of pH on acetic acid-oxidation by cell suspension.

Experimental conditions as for Fig. 4 except for use of 0.1 M sodium acetate instead of ethanol. Acetic acid-oxidation measured during the initial 30 minutes.

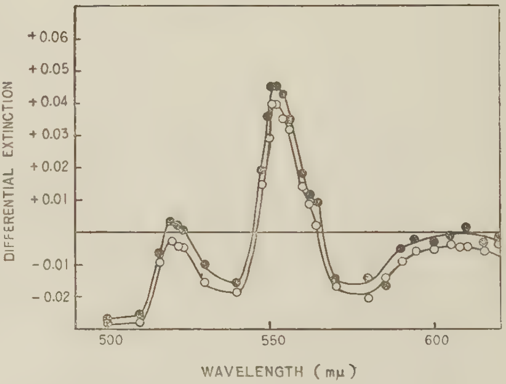


FIG. 6. Reduction of the cytochromes in the cells by ethanol and by acetaldehyde. The cell suspension was aerated at 30° for 12 hours. Reaction mixture, 2.0 ml. of aerated cell suspension, 1.0 ml. of 0.2 M phosphate buffer of pH 7.0 and 0.2 ml. of 0.5 M substrate. Total volume was adjusted with distilled water to 4.0 ml. Extinctions of the reaction mixture with and without substrate were compared. Reaction was started by addition of substrate, and optical densities were measured immediately after the addition. The densities hardly changed for about one hour. —●—, +ethanol; —○—, +acetaldehyde.

The cell suspension oxidized ethanol at almost the same rate between pH 6 and 8, and even at pH 5 there was only a little decrease in ethanol oxidation, as shown in Fig. 4. However, there was a sharp pH optimum at pH 7 for acetic acid-oxidation by the cell suspension, as shown in Fig. 5, and at pH 6 there was less than one third as much activity.

Interrelationship between Ethanol Oxidation and the Cytochromes—By microspectroscope, the cell paste with excess sodium dithionite showed a broad absorption band from 545 mμ to 565 mμ, and faint bands at around 590 mμ and 620–30 mμ.

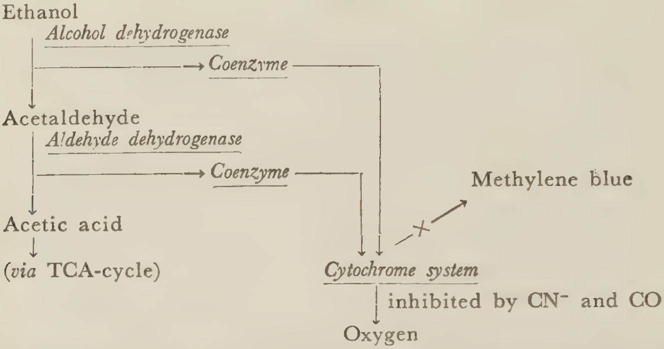
These absorption bands seemed to be the reduced α-absorption maxima of c-, b- and a-type cytochromes. When measured by the opal glass-method, the cytochromes present in cells which had previously been aerated for several hours were immediately reduced by addition of ethanol or acetaldehyde, as shown in Fig. 6.

DISCUSSION

Burton and Stadtman (*5*) purified DPN (diphosphopyridine nucleotide)-linked alcohol dehydrogenase and acetaldehyde dehydrogenase from *Clostridium kluyveri* and found that ethanol is oxidized to acetyl coenzyme A (Co A). A similar ethanol dehydrogenase has been purified from *Leuconostic mesenteroides* (*6*). The cell suspension of the strain of *Acetobacter* used in the present experiments as well as its cell-free extracts could rapidly oxidize ethanol to acetaldehyde, and one of the extracts could oxidize the latter to acetic acid. Therefore it is assumed that this bacterium oxidizes ethanol *via* acetaldehyde. These enzymes have not yet been purified, however, in separate form from this bacterium.

Ethanol oxidations by the cell suspension and the two kinds of cell-free extracts are strongly inhibited by cyanide and carbon monoxide, which are typical inhibitors of cytochrome oxidase. Moreover, like *Pseudomonas* cytochrome oxidase (*7*, *8*), cells could be reactivated from the carbon monoxide-inhibition to a great extent by light irradiation. Although ethanol is mainly oxidized by such a bacterial cytochrome oxidase, cyanide-inhibition is not prevented by the addition of methylene blue either with the cell suspension or cell-free extracts. This suggests that ethanol may be dehydrogenated by a dehydrogenase which is tightly bound to cellular particles and has an intimate relationship with the cytochrome system. In this way electrons would be passed from ethanol and from acetaldehyde to coenzymes also tightly bound to the same cellular particles, thus preventing by-pass of the electron transport system to methylene blue externally added. This assumption is also supported by the fact that all the ethanol-oxidative activity of the cell-free extracts can be precipitated by a high speed-centrifugation and this precipitated fraction can oxidize ethanol without being accelerated by addition of coenzymes. From the absorption spectra of the cells, the cytochrome system of this microorganism appears to be similar to that of *Pseudomonas aeruginosa* which has been clearly demonstrated to possess c-like, c_1-like, and b_1-like cytochromes and a_2-like cytochrome oxidase (*7–11*).

thanol, added as a substrate, is rapidly decomposed by the cells with a rapid consumption of oxygen and without evolution of carbon dioxide. This continues until the ethanol is almost completely decomposed to a nearly equimolar concentration of acetic acid. Since the cells can oxidize acetic acid in the absence of ethanol, however, the acetic acid itself begins to be catabolized immediately after the complete decomposition of ethanol. This indicates that the reduction of cytochromes in the cells by the additions of ethanol and acetaldehyde is directly related to the oxidation of these substrates to acetic acid as long as the ethanol was not yet completely been exhausted into the acid. The accumulation of acetic acid during ethanol oxidation can not be explained by the greater difference in optimal pH between the oxidation of ethanol to acetic acid and the oxidation of the latter. However, acetic acid-oxidation is much decreased if the pH of the reaction mixture becomes lower than 6. Two possible explanations for the great accumulation of acetic acid are shown in the following diagram:



where underlines shown that the enzymes and coenzymes are in cellular particle-bound form. First: the bound coenzymes present in the cells may all be used only for the oxidation of ethanol to acetic acid so that the enzyme responsible for the oxidation of the latter cannot be in function. Second: the oxidation of ethanol to acetic acid and the further metabolism of acetic acid may possibly be regulated, in some way unknown, by the particle-bound enzymes, coenzymes and cytochrome system.

Some bacteria were found to metabolize acetaldehyde *via* acetyl Co A to acetic acid, and the enzymes concerned with this pathway have been partially purified (*5*, *6*, *12–14*). DPN or TPN-linked aldehyde dehydrogenase functioning without Co A has been partially purified from baker's yeast (*15*, *16*) but not yet from bacteria.

SUMMARY

Oxidation of ethanol to acetic acid by *Acetobacter sp.* was studied in respect to the accumulation of much acetic acid as a metabolic intermediate of ethanol.

1. All components concerned with ethanol- and acetaldehyde-oxidizing activities were tightly bound to particulate fragments of the cells.

2. The oxidation of ethanol was remarkably inhibited by cyanide and carbon monoxide, indicating that a cytochrome oxidase was functional as a terminal oxidase in this ethanol oxidation. The inhibition by cyanide was, however, not removed in the presence of methylene blue at all, suggesting that there was an intimate relationship between the ethanol-oxidizing enzymes and the terminal electron-transferring system so that electrons liberated from ethanol might not be delivered to methylene blue.

3. As long as ethanol existed, the bacterial cells hardly metabolized acetic acid, even if the cells had acetic acid-oxidizing activity.

4. The cytochromes in the cells were rapidly reduced by the addition of ethanol and by acetaldehyde.

Mechanism of the accumulation of much acetic acid as a metabolic intermediate of ethanol was discussed based on these findings.

The author would like to express his thanks to Prof. K. Okunuki and Dr. T. Horio for their valuable guidance during this work.

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GLUTAMIC ACID FORMATION FROM γ-AMINOBUTYRIC ACID BY BACILLUS PUMILUS

II. ON THE PATHWAY OF GLUTAMIC ACID FORMATION

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In the previous report (*1*), the authors demonstrated in various cell preparations of *B. pumilus* 134-a a transaminase which acted strongly between γ-aminobutyric acid and glutamic acid. It was also shown that the bacterial cells actively metabolized γ-aminobutyric acid and β-formylpropionic acid, and accumulated L-glutamic acid and pyruvic acid (in the presence of arsenite) in the course of oxidation of γ-aminobutyric acid. Based on these observations, it was suggested that the major path of glutamic acid formation from γ-aminobutyric acid may involve the following sequence: γ-aminobutyric acid, β-formylpropionic acid, pyruvic acid, α-ketoglutaric acid, and glutamic acid.

The present paper reports some results on the pathway of glutamic acid formation by cell suspension of *B. pumilus* 134-a using γ-aminobutyric acid-1-C^{14}.

EXPERIMENTALS

γ-Aminobutyric Acid-1-C^{14}—Commercial γ-aminobutyric acid-1-C^{14} (Daiichi Pure Chemicals Co., Ltd.), which had about 500 mc./*M* of radioactivity and gave only one radioactive spot in the autoradiogram of paperchromatogram, was used. Its specific radioactivity was 3.89×10^4 c.p.m./μM in our G-M-counter.

Isolation and Determination of Specific Activity of Glutamic Acid Formed—Washed cells were incubated aerobically with γ-aminobutyric acid-1-C^{14} at 37° and were centrifuged. After being acidified with 2 drops of 3 *N* HCl, 1 ml. of the clear supernatant solution was dried *in vacuo* at 40°, and the sample was desalted by thrice extraction with a small volume of 85 per cent ethanol. The ethanol extract was evaporated to dryness and chromatographed two-dimensionaly in n-butanol—acetic acid—water (4 : 1 : 2) and then in phenol—water (100 : 20 *v/v*) containing final 0.1 per cent NH_3 after being spotted on Toyo No. 51 filter paper. The resulting glutamic acid spot was detected by the autoradiography. The glutamic acid spot was then eluted with about 1 ml. of water and its specific activity was determined by counting one aliquot and assaying another aliquot by a modification of the Moore and Stein ninhydrin procedure (*2*, *3*).

Location of Radioactivity in Labeled Glutamic Acid—The above mentioned ethanol extract was evaporated to dryness, dissolved in a small volume of water, streaked on Toyo No. 51 filter paper, and developed by phenol-water (100 : 20 *v/v*) containing final 0.1 per cent NH_3. The resulting glutamic acid band was cut out, and eluted with about 1 ml. of distilled water into the main compartment of a Warburg vessel. Three mg. of dried *E. coli* (Crookes strain) cells in 0.3 ml. of 0.1 *M* acetate buffer pH 4.8, which contained specific L-glutamic decarboxylase, was added from the side arm, and CO_2 evolved was absorbed into 0.2 ml. of 3 *N* NaOH in the center well. After the reaction mixture was deproteinized by the addition of ethanol to 67 per cent, and treated as described above, the ethanol extract was taken to dryness and chromatographed on Toyo No. 51 filter paper in phenol—water mixture described above (*4*). The γ-aminobutyric acid band was eluted and its specific activity determined by the procedure described above. This value means a specific activity of carbon 2~5 of labeled glutamic acid.

Isolation and Determination of Specific Activity of Pyruvic Acid Formed—Washed cells were incubated aerobically with γ-aminobutyric acid-1-C^{14} in the presence of 5×10^{-3} *M*/liter sodium arsenite at 37° and were centrifuged.

To 0.5 ml. of the supernatant solution, was added 0.5 ml. of 0.4 per cent 2,4-dinitrophenylhydrazine in 2 *N* HCl. After being allowed to stand overnight, the hydrazones were extracted with three 3 ml. portions of chloroform—ethanol (4 : 1). The combined solvent layers were extracted with 2 ml. of 10 per cent Na_2CO_3 (or 0.5 *M* phosphate buffer pH 7.8 in the case of the presence of β-formylpropionic acid derivative). The carbonate solution was washed with 1 ml. of chloroform-ethanol, and was extracted with 1 ml. of chloroform-ethanol after being acidified under cooling with 6 *N* HCl. This extract was evaporated to dryness, taken up in a small amount of ethyl acetate, and subjected to an ascending paper chromatography (*5*, *6*). The solvents used were n-butanol saturated with 3 per cent NH_3 (*7*), *n*-butanol—ethanol—0.5 N NH_4OH (7 : 1 : 2) (*8*), *n*-propanol—28 per cent NH_4OH—water (6 : 3 : 1), and phenol—water (100 : 20 *v*/*v*). The latter two solvents were used in the case of the presence of β-formylpropionic acid derivative (*1*).

The pyruvic acid derivative band was eluted with 2.5-10 ml. of distilled water and its specific activity was determined by counting one aliquot and assaying a second aliquot by the colorimetric procedure in 1 *N* NaOH at 440 mμ.

Detection of Isotopic Incorporation to Members of TCA-cycle—Washed cells were incubated aerobically with γ-aminobutyric acid-1-C^{14} and one of TCA cycle members at 37° for 25~40 minutes and were centrifuged. In the case of carbonyl acid the procedures were the same as for pyruvic acid described above. In the case of other organic acids 1 ml. of the clear supernatant solution was dried after being acidified with 3 *N* HCl, and the sample was desalted by thrice extraction with a small volume of ethanol. The ethanol extract was evaporated to dryness and chromatographed two-dimensionally in *n*-butanol—formic acid—water (4 : 1.5 : 1) (*9*) and then in phenol—water (100 : 20 *v*/*v*) (*10*) or ethanol—conc. NH_4OH—water (80 : 5 : 15) (*11*) after being spotted on Toyo No. 51 filter paper. The resulting radioactive spots were detected by autoradiography. The spots of organic acids other than carbonyl acids were then detected by spraying 0.1 par cent bromphenol blue in 95 per cent ethanol (*9*) or 0.05 per cent bromphenol blue aqueous solution (made acidic with 200 mg. citric acid per 100 ml.) (*12*) respectively. Detection of isotopic incorparation to each organic acid added was performed by comparison of the position and the shape of the radioactive spot with that of the acidic spot.

Preparation of cells, and determination of oxygen uptake was described in the previous report (*1*).

RESULTS

When γ-aminobutyric acid-1-C^{14} was oxidized by a cell suspension of *B.*

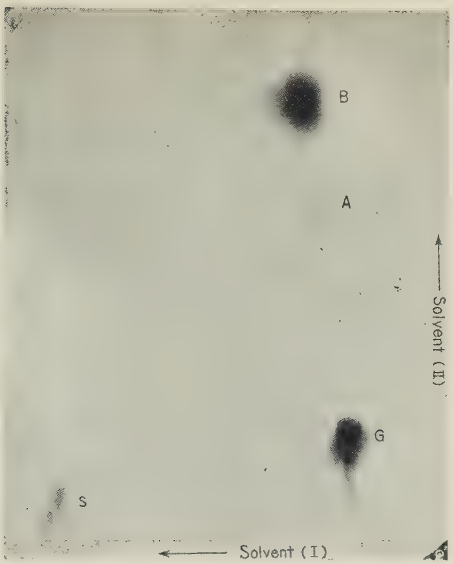


FIG. 1. Autoradiogram of oxidation products of γ-aminobutyric acid-1-C^{14} by *B. pumilus.* Reaction mixture contained cell suspension and 30 μM of γ-aminobutyric acid in a final volume of 2 ml. of 0.025 *M* phosphate buffer pH 7.0. Incubated aerobically for 140 minutes at 37°. Solvents in two-dimensional paper chromatography of the reaction mixture were *n*-butanol — acetic acid — water (4 : 1 : 2) (I) and phenol — water (100 : 20 *v/v*) containing final 0.1 per cent NH$_3$ (II).

O: original point, B: γ-aminobutyric acid.
G: glutamic acid, A: alanine, S: succinic acid.

TABLE I

Specific Radioactivity of Glutamic Acid and Pyruvic Acid Formed in the Oxidation of γ-Aminobutyric Acid-1-C^{14}

	Specific radioactivity	Ratio[3]
γ-Aminobutyric acid (substrate)	3.89×10^4 *c.p.m./*μM	100
Glutamic acid[1]	1.70 ,,	44
,, —2∼5-C	0.0184 ,,	0.47
,, —1-C	1.68 ,,	43
Pyruvic acid[2]	1.83 ,,	47

1) Contents of reaction mixture and conditions of reaction were the same as in Fig. 1.

2) Reaction mixture contained cell suspension, 5 μM of γ-aminobutyric acid-1-C^{14} and 5×10^{-3} *M* NaAsO in a final volume of 2 ml. of 0.025 *M* phosphate buffer pH 7.0. Incubated aerobically for 35 minutes at 37°.

3) Specific activity relative to that of the added substrate.

pumilus, an autoradiogram of two-dimensional paperchromatogram of reaction mixture was obtained, which was reproduced in Fig. 1. This autoradiogram shows the existence of γ-aminobutyric acid (γ-ABA), glutamic acid, alanine, and succinic acid as completely separte spots. The latter two spots after being eluted with water, were identified by co-chromatography with chromatographically related known materials respectively.

Table I shows the specific radioactivites of glutamic acid and pyruvic acid formed in the course of the oxidation of γ-ABA-1-C^{14} by cell suspension, both of which are about a half of that of the substrate, γ-ABA-1-C^{14}. The table also shows that the glutamic acid isolated was almost exclusively labeled in the α-carboxyl carbon. Since these date suggested the glutamic acid formation through the normal forward reaction of the Krebs cycle, the relationships between various organic acids in the Krebs cycle and glutamic acid formation from γ-ABA were analyzed by the tracer incorporation and isotopic competion method. As shown in Table II, the addition of each acid listed resulted in lowering of specific activity of glutamic acid or pyruvic acid (in the presence of arsenite) formed from γ-ABA-1-C^{14}, and the isotopic incorporation to each acid added was detected in all cases except for oxalacetic acid and isocitric acid.

Table II

Isotopic Incorporation to Added Substance and its Effect on the Specific Radioactivity of Product in the Oxidation of γ-Aminobutyric Acid-1-C^{14}

Exp.	Organic acid added.	NaAsO$_2$	Isotopic incorporation	Specific radioactivity of product
1	None	+		1.91 × 10^4 *c.p.m.*/μM (pyruvic)
	β-Formylpropionic acid	+	+	1.61 ,, (,,)
2	None	+		1.74 ,, (,,)
	L-Malic acid*	+	+	0.69 ,, (,,)
	Fumaric acid	+	+	1.55 ,, (,,)
	Succinic acid	+	+	1.58 ,, (,,)
3	None	−		1.54 ,, (glutamic)
	α-Ketoglutaric acid	−	+	1.08 ,, ( )
	Isocitric acid	−		1.28 ,, ( )
	Oxalacetic acid	−	−	0.96 ,, ( )
4	None	−		1.83 ,, ( )
	β-Formylpropionic acid	−	+	1.23 ,, ( )
	Isocitric acid*	−	−	1.47 ,, ( )

Reaction mixture contained cell suspension, 5 μM of γ-aminobutyric acid-1-C^{14}, 5 μM (* : 10 μM) of organic acid as indicated, and 5×10^{-3} M NaAsO$_2$ in 2 ml. of 0.01 M phosphate buffer pH 7.0. Incubated aerobically for 25–40 minutes at 37°.

DISCUSSION

In the previous paper (*1*), it was demonstrated that β-formylpropionic acid was an intermediate in the metabolism of γ-ABA via transamination. Isotopic incorporation to β-formylpropionic acid from γ-ABA-1-C^{14} in the presence of arsenite, which inhibited almost completely the metabolism of γ-ABA via transamination, suggests the occurrence of the following oxidative degradation of γ-ABA:

$$NH_2-CH_2-CH_2-CH_2-COOH + O \longrightarrow CHO-CH_2-CH_2-COOH + NH_3$$

By the observation that the radioactivity of γ-ABA-1-C^{14} goes exclusively to carbon 1 of glutamic acid formed, and that the specific activity of glutamic acid and pyruvic acid formed is about a half of that of the substrate, γ-ABA-1-C^{14}, the following pathways could probably be excluded:

1) Reversal of the decarboxylation of glutamic acid.

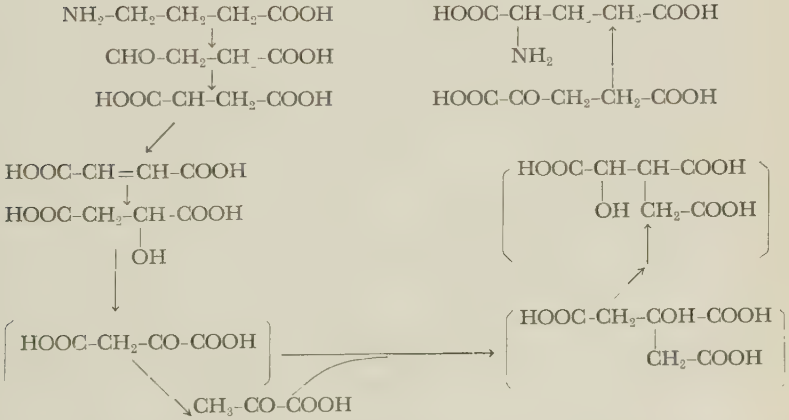
2) The pathway involving direct carboxylation of succinic acid, succinyl·CoA, or β-formylpropionic acid to form α-ketoglutaric acid.

3) The pathway involving the splitting of γ-ABA or its degradation product to form two moles of acetate with subsequent formation of α-ketoglutaric acid through the normal forward reaction of the Krebs cycle or the modified Krebs cycle (*13–15*).

4) The pathway containing the formation of oxalacetate without intermediate formation of symmetric four carbon compouns with subsequent formation of α-ketoglutaric acid through the normal forward reaction of the Krebs cycle.

DIAGRAM 1

Probable Pathway of Glutamic acid formation from γ-aminobutyric acid by *Bacillus pumilus* 134-a



On the other hand, this observation suggests a pathway involving the formation of oxalacetate via symmetric four carbon compounds with subsequent formation of α-ketoglutaric acid through the normal forward reaction of the Krebs cycle, in which oxalacetate formed is labeled in both α- and β-carboxyl carbons and each specific activity is a half of that of the substrate, γ-ABA-1-C^{14}, and then the resulting α-ketoglutaric acid becomes labeled exclusively in its α-carboxyl group, because of the loss of α-carboxyl carbon of oxalacetate in the subsequent decarboxylation of oxalosuccinate and the metabolic asymmetry of citric acid (*16*).

This is consistent with the following sequence suggested in the previous paper: γ-ABA, β-formylpropionic acid, pyruvic acid, α-ketoglutaric acid, glutamic acid.

Moreover, this possibility seems to be partially supported by the tracer incorporation and isotope competition experiments as shown in Table II.

The pathway of γ-aminobutyric acid to glutamic acid could be expressed as shown in diagram 1.

The failure to demonstrate the isotopic incorporation to oxalacetate and isocitrate from γ-ABA-1-C^{14}, and the reactivity of citrate and *cis*-aconitate in cells is by no means conclusive evidence against the possible participation of these substances in metabolic process, because of the existence of permeability barriers. However, it also seems to be possible that α-ketoglutaric acid may be formed via a direct condensation of pyruvic acid with acetyl-CoA, which can account for all date described here and in the previous paper, although this reaction has never been demonstrated in a cellular system.

SUMMARY

1. When γ-aminobutyric acid-1-C^{14} was incubated aerobically with a cell suspension of *B. pumilus* 134-a, glutamic acid isolated was labeled almost exclusively in the α-carboxyl carbon, and the specific radioactivities of this acid and pyruvic acid formed in the presence of arsenite were about a half of that of the substrate, γ-aminobutyric acid.

2. From the tracer incorporation and isotope competition experiments it was suggested that β-formylpropionic acid, succinic acid, fumaric acid, L-malic acid, pyruvic acid, and α-ketoglutaric acid might be intermediates in the glutamic acid formation from γ-aminobutyric acid by the cells.

3. The results obtained here and in the previous paper are consistent with oxidation and transamination of γ-aminobutyric acid into β-formylpropionic acid, with subsequent formation of succinic acid, which is converted into glutamic acid through the normal forward reaction of the Krebs cycle.

The authors are indebted to Prof. S. Akabori, University of Osaka, and Dr. T. Ogawa of our company, for the helpful criticism and encouragement during the course of this work.

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STUDIES ON ENZYMIC NITRITE REDUCTION

II. SEPARATION OF NITRITE REDUCTASE TO PARTICULATE AND SOLUBLE COMPONENTS

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(Received for publication, February 23, 1959)

In the preceding paper of this series (*1*) it was reported that electron transport system functioning in the reduction of nitrite in halotolerant micrococcus strain 203 was similar to the electron transport system to oxygen through the cytochrome system. It is well known that cytochrome oxidase acting on the terminal position of the latter exists in a particulate fraction. So it is interesting to explore the state of existence of nitrite reductase acting on the terminal position of the electron transport system to nitrite. Dialyzed cell suspension lyzed by osmotic pressure change, however, was so viscous that centrifugal separation was difficult. Deoxyribonuclease treatment of dialyzed cell suspension considerably decreased its viscosity, and made easier the centrifugal fractionation. After the ultracentrifugal separation of dialyzed cell suspension to supernatant and particulate fractions, NiR* activity remarkably decreased in each fraction but the activity was recovered when both fractions were recombined. It seems that nitrite reductase consisted of at least two factors, one of which is soluble and the other is particulate. Without the DNase treatment the centrifugal separation of the soluble factor from particulate factor was imcomplete in spite of several washings of the particulate fraction.

This report deals with the separation, distribution, partial purification and properties of these factors.

MATERIALS AND METHODS

Microorganism—A halotolerant Micrococcus strain 203 was cultured aerobically, harvested and lyophilyzed as described in the communication by Kono *et al.* (*2*). Lyophilyzed cells were suspended at the concentration of 80 mg./ml. in a phosphate buffer (pH 6.8, *M*/100) which contained crystalline DNase I at the concentration of 10 μg./ml., and dialyzed overnight against phosphate buffer (pH 6.8, *M*/100) in a cellophane turbing at 3°. After dialysis, the cell suspension was centrifuged at 20,000×g for 30 minutes and the red supernatant and the precipitate were obtained.

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* Following abbreviations were used: NiR, nitrite reductase; HdR., hydroxylamine reductase; PMSH, reduced phenazine methosulfate; MbH_2, reduced methylene blue; PF, particulate factor; SF, soluble factor; DNase, deoxyribonuclease.

Purification of Soluble Factor—To the red supernatant phosphate buffer (pH 6.8) and sodium chloride were added to the final concentration of 0.02 M and 0.3 M, respectively, and then cold acetone was added. The yellow precipitate obtained between 40 and 50 volume per cent of cold acetone was collected, dissolved in cold distilled water and lyophilyzed. The lyophilyzed enzyme preparation (1.0 g.) was dissolved in 100 ml. of cold distilled water and acetate buffer (pH 5.5) was added to the final concentration 0.01 M. And to the enzyme preparation 20 ml. of calcium phosphate gel* (50 mg. dry weight/ml.) was added. The gel was centrifuged off and washed twice with 100 ml. of $M/30$ phosphate buffer (pH 6.8) and the enzyme was eluted twice from the gel with 50 ml. and 25 ml of 0.1 M phosphate buffer (pH 6.8) respectively. The eluates were combined and stored in a deep freezer at −15°.

Purification of Particulate Factor—On the other hand, the precipitate obtained by the centrifugation of dialyzed cell suspension was washed twice with cold distilled water and stored in a deep freezer, and the frozen precipitate was thawed, suspended in cold distilled water and centrifuged at 2000×g for 10 minutes. The turbid supernatant thus obtained was centrifuged at 20,000×g for 30 minutes and the precipitate was washed twice with cold distilled water.

The reddish particulate fraction was lyophilyzed or stored in a deep freezer at −15°. By these procedures a particulate factor free from the soluble factor was obtained.

Without using the ultracentrifugation the particulate fraction contained in 20,000×g supernatant could be separated from the soluble factor by calcium phosphate gel treatment in the condition described above. Particulate factor was found exclusively in gel unadsorbed fraction, and precipitated by the addition of solid ammonium sulfate at the concentration of 50 per cent saturation. The precipitate was suspended in cold phosphate buffer (pH 6.8, 0.02 M) and used in the experiments.

Assay of Enzymes—Method of assay of nitrite reductase was described in the previous paper (*1*). Activity of cytochrome oxidase was measured by the method using *p*-phenylenediamine as described by Yamada and Asano (*3*).

Determination of Protoheme—Protoheme was determined by the method described by F. Moss (*4*).

RESULTS

Distribution of Soluble and Particulate Factors—Distribution of the soluble and particulate factors and related enzymes after centrifugal fractionations as studied. As shown in Table I, the soluble factor was found exclusively in 100,000×g supernatant and it was known that hydroxylamine reductase was found exclusively in this fraction too (*5*). The particulate factor was found in 1,000–20,000×g particle. Protoheme and cytochrome oxidase were found in the same fraction.

Nature of Particulate and Soluble Factors—The factors were proved to be of protein nature by heat treatment and protease treatments (Table II). Particulate factor was more sensitive against heat treatment than soluble factor; it lost 45 per cent of the activity by 70° 5 minutes in 0.05 M Na_2HPO_4. The particulate factor was also inactivated by crystalline proteinase from *B. subtilis* and trypsin.

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* Method of the preparation of the calcium phosphate gel was as described in a previous paper (*1*).

TABLE I

Distribution of Particulate Factor, Soluble Factor and Related Components after Centrifugal Fractionation

	Total nitrogen	Soluble factor¹⁾	Particulate factor¹⁾	Protoheme	Cytochrome oxidase²⁾
Whole cell	211 mg.	352 units	485 units	72.0 μg.	192 units
0-1,000×g precipitate	14	0	2	—	0
1,000-20,000×g precipitate	54	0	414	69.0	112
20,000-100,000×g precipitate	3	0	14	0	36
100,000×g supernatant	125	325	20	1.9	0
Sum of each fraction	196	325	450	70.9	148
Recovery (per cent)	93.0	92.5	92.7	98.7	72.2

1) The amount of soluble and particulate factor was separately measured by the nitrite reduction of PMSH-NiR system under the saturation of the other factor. The unit of each factor shows the amount of nitrite reduced (μ moles) per hour.

2) The unit of cytochrome oxidase is defined as the amount of the enzyme which take up 1 μl. of oxygen per hour.

TABLE II

Protein Nature of the Soluble and Particulate Factors

Particulate factor	Soluble factor	Nitrite reduced (μM/75 min.)
+	—	0.08
+	+	2.63
+	+ (100°, 10 mins. treated)	0.42
+	+ (bacterial proteinase treated*)	0.09
+ (100°, 10 mins. treated)	+	0.00

* Crystalline proteinase from *B. subtilis* and phosphate buffer (pH 6.8) were added to final concentration of 2 mg./ml. and 0.02 M respectively. Incubation was carried out at 30°, for an hour.

Participation of Particulate and Soluble Factors in Denitrification—The requirement of both factors for denitrification was observed not only by the determination of nitrite but also by gas production (Fig. 1).

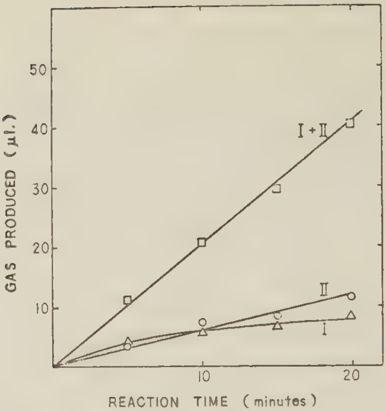


FIG. 1. Participation of particulate and soluble factors in denitrification.

I. 20,000×g supernatant.

II. The acetone precipitated fraction (50–70 per cent) of ultrasonic extract from 20,000×*g* precipitate.

A fraction rich in the particulate factor was prepared by the ultrasonic method described in the previous report (*1*), and 20,000×g supernatant was used as a fraction rich in the soluble factor. About two-fold activation of nitrogen gas production was observed by combining the two fractions.

Purification of Soluble Factors—Purification of the soluble factor was carried out by a procedure similar to that of hydroxylamine reductase (*5*). As shown in Table III, the activity of the soluble factor was almost parallel with that of HdR at least until this step of the purification.

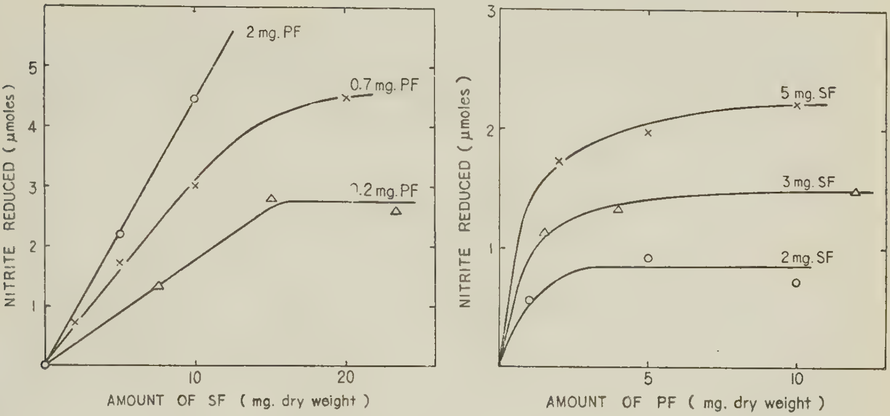
TABLE III

Purification of Soluble Factor

Fractions	Nitrite reduced* (*μM/hour*)	Hydroxylamine reduced (*μM/hour*)
Acetone 0–40 per cent	0	0
,, 40–50 per cent	1.75	5.36
,, 50–67 per cent	0	0
Calcium phosphate gel unabsorbed	0.32	0
Eluted by 0.03 *M* phosphate buffer (pH 6.8)	0.29	0
Eluted by 0.1 *M* phosphate buffer (pH 6.8)	1.69	6.66

* Saturated amount of PF was added.

Relation between the Quantities of Soluble and Particulate Factors and NiR Activity—As shown in Fig. 2 (a) and Fig. 3 (a), when a fixed amount of the particulate factor and varied amounts of the soluble factor were used, PMSH-NiR system was saturated by a smaller amount of the soluble factor than MbH_2-NiR system. Fig. 2 (b) and Fig. 3 (b) show the saturation curve of the particulate factor. MbH_2-NiR system was saturated by a smaller amount of the particulate factor than PMSH-NiR system. When NiR activity was

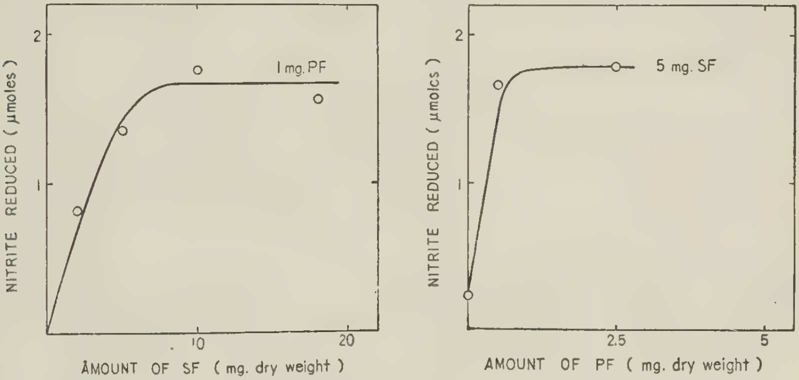


a b

FIG. 2. Effect of soluble factor and particulate factor on nitrite reductase activity (PMSH-NiR system).

(a) The fixed amount of PF and varied amounts of SF were added.

(b) The fixed amount of SF and and varied amounts of PF were added.



a b

FIG. 3. Effect of soluble factor and particulate factor on nitrite reductase activity (MbH_2-NiR system).

(a) The fixed amount of PF and varied amounts of SF were added.

(b) The fixed amount of SF and varied amounts of PF were added.

measured with or without inhibitors under the saturation of soluble factor, the inhibition observed may be due mainly to that for the activity of particulate factor. By the same method the inhibition for the activity of soluble factor could be measured under PF saturation.

Inhibition of the Activity of Soluble and Particulate Factors by Metal Inhibitors—The activities of soluble and particulate factors were inhibited by metal inhibitors (Table IV, Table V). In the PMSH-NiR system, PF activity was inhibited strongly by KCN and Fe^{++}-chelators, but the SF activity was less inhibited by the same reagents.

TABLE IV

Inhibition of PMSH-NiR System

Inhibitors		Inhibition (*per cent*)	
	Concentration (M)	Activity of PF (SF 15 mg., PF 0.1 mg.)	Activity of SF (SF 3 mg., PF 3 mg.)
KCN	10^{-3}	86	42
o-Phenanthroline	2×10^{-3}	54	23
α,α'-Dipyridyl	2×10^{-3}	42	16
Sodium diethyl dithiocarbamate	2×10^{-3}	17	28
Salycyl aldoxime	10^{-3}	28	26
Allyl thiourea	10^{-3}	16	8
Carbon monoxide (light)	0.1 atm.	15	20
,, (dark)	0.1 atm.	17	21
Hydroxylamine	10^{-3}	0	0

TABLE V

Inhibition of MbH_2-NiR System

Inhibitors		Inhibition (*per cent*)	
	Concentration (M)	Activity of PF (SF 10 mg., PF 1 mg.)	Activity of SF (SF 5 mg., PF 5 mg.)
KCN	10^{-3}	79	69
o-Phenanthroline	2×10^{-3}	39	25
Sodium diethyl dithiocarbamate	2×10^{-3}	10	46
Salycyl aldoxime	10^{-3}	5	30
Allyl thiourea	10^{-3}	7	26
Carbon monoxide (light)	0.1 atm.	19	24
,, (dark)	0.1 atm.	45	45
Hydroxylamine	10^{-3}	85	100

In the MbH_2-NiR system the same tendency was also observed. Moreover, the SF activity was much inhibited than the PF activity by Cu^{++}-chelators. Both the PMSH-NiR and MbH_2-NiR systems were inhibited by carbon monoxide but only in the latter case the activity was partly recovered by the light. Hydroxylamine inhibited the MbH_2-NiR system but not the PMSH-NiR system.

DISCUSSION

In the terminal electron transport system, it is known that soluble and particulate fractions are cooperating together, for example in *Mycobacterium phlei* (*6*) and in microsomal electron transport system with cytochrome c in animal tissues (*7*).

In denitrification, H. Iwasaki and T. Mori discovered that the denitrifying activity measured by dimethyl-*p*-phenylene diamine-nitrite system was separated into two soluble components by ammonium sulfate fractionation (*8*).

It is interesting that the separation of the two components was found in different denitrifying systems which have different nitrite metabolizing mechanisms and different electron donors. The following two possibilities may be considered for the mechanism of action of the two factors: (1) One of them acts as an electron carrier and the other factor acts as the nitrite reductase. (2) Production of nitrogen gas from nitrite is a three-electron reduction and needs the formation of nitrogen-nitrogen bond. So nitrite reduction to molecular nitrogen may be divided into at least two steps. And each step may be catalyzed by a different enzyme. The latter possibility was supported by the inhibition study of SF and PF, which suggested that the different non-heme metal was functioning in each factor. But to draw the final conclusion, further purification of two factors and kinetic and metabolic studies may be necessary.

As described briefly in previous papers, MbH_2-NiR system contains a cytochrome-like intermediary electron carrier (3rd factor) which is hydroxylamine sensitive and photoreversibly CO-binding. Separation of the 3rd factor from SF and PF is now under progress.

SUMMARY

1. Nitrite reductase of halotolerant *Micrococcus* strain 203 was separated to soluble and particulate components by centrifugation. Each fraction did not show the NiR activity by itself, but the activity appeared when both fractions were recombined.

2. DNase treatment made the separation of both factors easier.

3. Both factors were of protein nature.

4. The properties of each factor were studied under the condition of saturation of the other factor.

5. Different metals may be functioning in soluble and particulate factors.

6. Working mechanism of both factors were discussed.

7. Participation of hydroxylamine sensitive and photoreversibly carbon monoxide binding factor in the MbH_2-NiR system was discussed.

The auther wishes to thank Prof. F. Egami for his kind encouragement and discussion.

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STUDIES ON THE RIBONUCLEOPROTEIN PARTICLES

II. AN ELECTROPHORETIC STUDY ON THE MICROSOMAL RIBONUCLEOPROTEIN PARTICLES ISOLATED FROM RAT LIVER*

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(Received for publication, February 27, 1959)

Microsomal ribonucleoprotein (RNP) particles were first isolated by Littlefield *et al.* (*1*) from rat liver and the importance of these particles in the synthesis of cell protein has been demonstrated. The extreme unstability of the particles, however, has been a hindrance to detailed physicochemical investigation. Petermann (*2*, *3*) has found that the rat liver supernatant has a stabilizing effect on these particles and made use of it to the centrifugal and electrophoretical studies on them. The present authors have reported an electronmicroscopical observation (*4*), Ribonuclease (RNase) activity (*5*, *6*) and some physicochemical properties of these particles (*7*). It is the purpose of the present communication to report an electrophoretical study on the RNP particles.

EXPERIMENTAL

The RNP was prepared from the rat liver microsomes by the method of Littlefield *et al.* (*1*), using No. 30 roter of Spinco Model L ultracentrifuge. Ten gm. portions of liver were minced in 30 ml. of ice cold 0.25 *M* sucrose and homogenized at 0° in a glass homogenizer. The homogenate was centrifuged at 0° for 10 minutes at 18,000×*g*, and the precipitate was discarded. The supernatant fluid was sedimented at 0° for 60 minutes at 78,000×*g* (R average). The soluble fraction (microsomal supernatant) was decanted, and the pellet (microsomes) was treated with sodium deoxycholate, and after dilution with cold water it was sedimented at 78,000×*g* for 160 minutes. The supernatant fluid was sucked carefully from around the loosely packed sediment of the RNP. These sediments were collected and resuspended in the Miller-Golder's buffer (*8*) of pH 7.0 and ionic strength 0.1, or in the "buffered dialysate" (see below) and centrifuged at 20,000×*g* for 30 minutes. The clear supernatant was carefully pipetted out and used as the RNP solution. When the amount of deoxycholate was optimal, this solution was clear and was hardly contaminated with phospholipids. It contained about 40 per cent RNA and 60 per cent protein on a dry weight bases.

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* The outline of the paper was read at the International Symposium on Enzyme Chemistry at Tokyo and Kyoto 1957.

The RNP samples for electrophoretic analysis were prepared in the two ways. (i) The RNP solution was dialysed overnight against the Miller-Golder's buffer under continuous stirring, and after adjusting its final concentration to about 1 per cent it was observed by an electrophoretic apparatus. All the RNP samples excepting for Fig. 1a were prepared in such a manner. (ii) As it has been reported by Petermann (*2*, *3*) that a dialysable material present in the soluble fraction of rat liver effectively preserves the RNP, the microsomal supernatant was dialysed overnight against about ten times volume of the Miller-Golder's buffer in the cold and the RNP was prepared in this "buffered dialysate". Therefore this "dialysate" correspounds to "buffer-SF" of Petermann (*2*, *3*).

The RNP was used without further purification, because, when properly prepared, it was possible to prepare the RNP contaminated with less than 5 per cent phospholipids and also the purification procedures were very time consuming. In order to prevent the autodegradation of the RNP, it was essential to perform all the procedures in a hurry and at low temperature (*0–5*°).

Electrophoresis—The Hitachi Model HTD-1 type of electrophoretic apparatus was exclusively used. Electrophoresis was carried out in the cold (at 4∼5°), photographs were taken at every ten minutes interval and the ascending and descending electrophoretic mobilities at 0° (U_0) were calculated from the conductivity of the solvent at 0°, which was determined with a wheatstone bridge, a conductivity cell and a low frequency oscillator adjusted at 1,000 cycles per second.

RNase free from protease was obtained from the Wako Pure Chem. Industries, Osaka, and the crystalline trypsin was obtained from the Worshington Biochem. Co. These enzymes were previously dissolved in the buffer and were added to the RNP solution before use at a final concentration of 0.01 per cent.

After electrophoretic analysis, the samples were pipetted out of the cell and analysed by the Schmidt-Thannhauser's method slightly modified by Littlefield *et al.* (*1*). RNA content of the samples was determined from the optical density at 260 mμ by using an extinction coefficient of 34.2 per mg. per ml. per cm., and the protein was dried at 110° for 5 hours and dry weight was weighed. Phospholipids were ashed with perchloric acid and inorganic phosphate was determined by Allen's method (*9*).

RESULTS

A. Electrophoretic Patterns of the RNP Prepared in the "buffered Dialysate"—In Fig. 1-a is shown an example of the typical electrophoretic patterns of the RNP which was freshly prepared in the "buffered dialysate" after the dialysis of 3 hours. The large fast boundaries represent the RNP (component 2) which had an average ascending and descending mobility (U_0) of -11.6 ± 0.3 and $-11.0\pm0.3\times10^{-5}$ cm^2. per sec. per volt in this "buffered dialysate" of ionic strength 0.10 at pH 7.0 (avarage of six examples). These values are in good agreement with the mobilities of the RNP prepared with streptomycin (*10*).

The main peaks are, however, accompanied with a small component (component 3) with slower mobility, of which avarage ascending and descending mobility (U_0) were -9.6 ± 0.7 and $-8.8\pm0.6\times10^{-5}$ cm^2. per sec. per volt respectively. This conponent probably corresponds to the turbid component

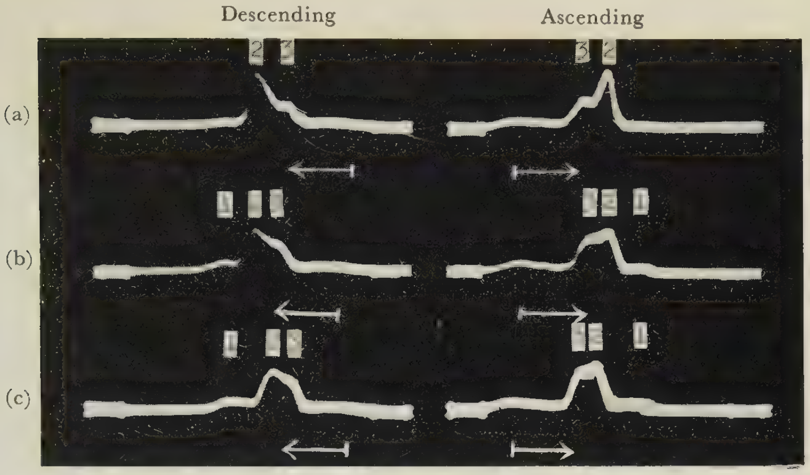

FIG. 1. Electrophoretic patterns of the RNP particles which were prepared in the "buffered dialysate" (a) or in the Miller-Golder's buffer ((b) and (c)).

(a) The RNP isolated from rat liver microsomes was prepared in the "buffered dialysate" of the microsomal supernatant, and analysed electrophoretically in the "buffered dialysate", pH 7.0, ionic strength 0.1, in a field of 6.2 volts/cm. The RNP solution for Fig. 1-a was far fresher than that of Petermann's because the electrophoresis was carried out within 36 hours after the sacrifice of the rats or within 9 hours after the deoxycholate treatment. Though the patterns of the RNP (2) are accompanied with a small component (3), there is neither free RNA nor any other component with smaller mobility.

(b) and (c) The RNP was isolated as in the Fig. 1-a and dialysed against the Miller-Golder's buffer, pH 7.0, ionic strength 0.1 for 12 hours (b) and 36 hours (c) respectively and analysed electrophoretically under the similar conditions as in Fig. 1-a.

T of the purified RNP reported by Petermann (*3*). As to the origin of this component, there are two possibilities. The one is that it represents the fragments of the microsomes which were destructed with deoxycholate and the other is that the component is the partially dissociated ribonucleoprotein particles. The former estimation receives support from our observation that the prominence of this component is roughly proportional to the degrees of the contamination of the phospholipids. The latter possibility is supported by the fact that the electrophoretic patterns of the RNP which is prepared in the Miller-Golder's buffer show degradation of the component 2 with compensatory increase of the component 3, as shown in Fig. 1 b and c.

Neither free RNA nor any other slower components such as ferritin could be detected.

B. Electrophoretic Patterns of the RNP Prepared in the Miller-Golder's Buffer—In Fig. 1-b and 1-c are shown the electrophoretic patterns of the RNP prepared in the Miller-Golder's buffer of ionic strength 0.1 and pH 7.0 Comparing b and c with a, it is apparent that the peak of the RNP (component 2) remarkably decreased, while the component 3 became more prominent than in Fig. 1-a suggesting the possibility of transformation from the component 2 to 3. Such degradation seems to proceed with dialysis till the electrophoretic patterns of the component 2 and 3 became equal in size and appeared like a continuous peak as shown in Fig. 1-c. Average ascending and descending mobility (U_0) of the component 2 therefore decreased than in Fig. 1-a and were -10.9 ± 0.4 and $-10.3\pm0.4\times10^{-5}$ cm^2. per sec. per volt. respectively (average of nine samples). The apperance of a small faster peak (component 1) was occasionally noted, which probably corresponds to the free RNA released out of the RNP particles (see Fig. 2).

As the microsomal RNP particles have RNase activity in themselves (5. 6), and as the RNA content in this sample was always somewhat less than that in the RNP prepared in the " buffered dialysate ", the degradation is probable due to their own RNase activity, and a RNase inhibitor present in the " buffered dialysate " seems to be responsible for the stablility of the particles in this solution (*5*, *6*).

C. Electrophoretic Patterns of the Autodegraded RNP Particles—The RNP solution was incubated for a definite interval at 37° without adding enzyme, cooled again and the solution was analysed by the electrophoretic method. As shown in Figs. 2-a and 2-b, the most remarkable change is the appearance of a small peak (component 1) with average ascending and descending mobility of -16.3 and -13.9×10^{-5} cm^2. per sec. per volt. As the average mobilities of yeast RNA were -16.4 and -14.8×10^{-5} cm^2. per sec. per volt under the similar experimental conditions, it probably corresponds to the free RNA released out of the RNP particles as in the case of Figs. 1-b and 1-c.

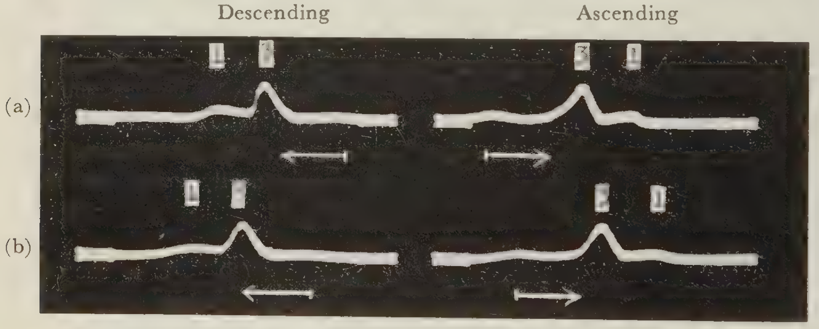

FIG. 2. Electrophoretic patterns of the autodegraded RNP particles.
The RNP was incubated at 37° for 15 (a) and 30 minutes (b) respectively without adding enzyme and observed by electrophoresis. Appearance of a new peak (1) of which electrophoretic mobility is almost equal to that of free RNA is noted.

It is also interesting that the mobility and size of the component 2 further decreased with incubation till the component 2 was completely transformed to the component 3, of which average ascending and descending mobility were -10.0 and -8.9×10^{-5} cm². per sec. per volt respectively.

As reported by the author (5, 7), when the RNP is incubated at 37° without adding enzyme, acid soluble RNA increases gradually and after 20 minutes, it amounts to about 20 per cent of total RNA, while protein of the RNP shows hardly any degradation. From these findings it seems probable that the electrophoretic mobility of the RNP particles depends to some extent upon the RNA content of the particles.

D. Enzymatic Degradation of the RNP Particles—The RNP particles are easily digested either with pancreatic RNase or with trypsin (4, 5, 6). When 0.5 per cent RNP solution was inclubated with an aliquot of 0.01 per cent RNase at 37°, about 85 per cent RNA of the RNP was depolymerized within 20 minutes (in this report "depolymerize" means "impossible to precipitate with an aliquot of *N* perchloric acid"). As the turbidity increase accompanied with such an incubation (7) made it always impossible to perform electrophoretic analysis, RNase was added in the cold at a final concentration of 0.01 per cent and observed electrophoretically without previous incubation at 37°. Even under such a condition, about 20 per cent of RNA was found depolymerized.

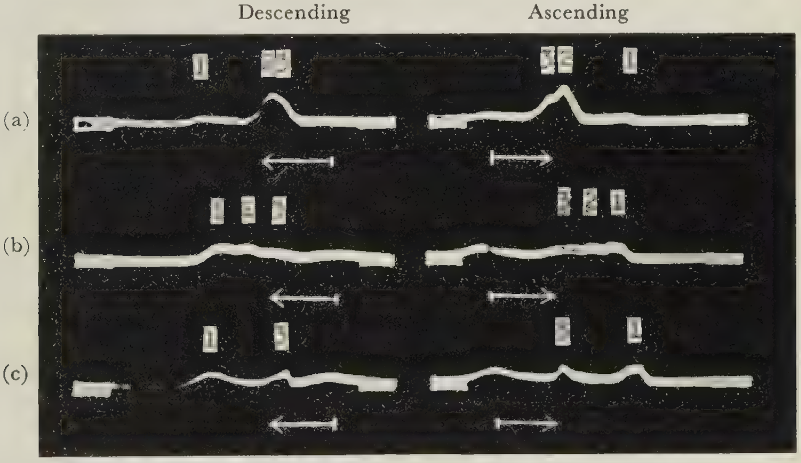

FIG. 3. Electrophoretic patterns of the RNP treated with the RNase (a) and the trypsin (b, c).

(a) To the RNP solution, RNase was added in the cold at a final concentration of 0.01 per cent and the mixture was analysed by electrophoresis without previous incubation at 37°.

(b) and (c) Electrophoretic patterns of the RNP incubated with the trypsin at 37° for 5 (b) and 20 minutes (c) respectively. Destruction of the RNP into smaller components is remarkable.

Electrophoretic patterns (Fig. 3a) are similar to Fig. 2 and are characterized by the appearance of free RNA (in this case ascending and descending mobility were -16.4 and -13.8×10^{-5} cm². per sec. per volt respectively) and the decrease in the mobility of the component 2 (the mobilities were -9.0 and

-7.7×10^{-5} cm². per sec. per volt).

It has been reported by the authous (*5*, *7*) that by the incubation of the RNP (0.3–0.5 per cent) with trypsin (0.005 per cent) at 37° for 20 minutes about 40 per cent of protein and 30–40 per cent of RNA were depolymerized and that the RNP particles were destructed with trypsin into such smaller fragments as no more sedimentable by the centrifugation at $105{,}000 \times g$ for 120 minutes.

Fig. 3-b, and 3-c are the electrophoretic patterns of the RNP treated with trypsin at 37° for 5 and 20 minutes respectively. Fig. 3-b shows that the incubation for 5 minutes was sufficient for the destruction of the RNP peak into about three components. The average ascending and descending mobility of the fastest one were -15.7 and -13.7, and those of the intermediate one were -12.0 and 10.9×10^{-5} cm². per sec. per volt respectively. After incubation for 20 minutes, the second peak disappeared as shown in Fig. 3-c, and the slowest one became more prominent, of which mobilities were -7.4 and -5.5×10^{-5} cm². per sec. per volt. The patterns of Fig. 3-c had the elevated δ and ε boundaries, which were stationary throughout the electrophoresis.

These results suggest that trypsin destructs the RNP particles into smaller fragments and then dissociates them to the faster (RNA) and slower component (protein ?).

DISCUSSIONS

In view of evidence that the RNP particles represent a stage in the process of protein biosynthesis, the structure of these particles is of considerable biological interest.

It has been generally admitted that the viral nucleoprotein has a definite structure. For example, tobacco mosaic virus (*11*), turnip yellow mosaic virus (*12*) and T_2 bacteriophage (*13*), etc. are reported to have their nucleic acid in the core of their particles completely covered by protein. Nucleotropomyosin (*14*) and deoxyribonucleoprotein of chromosomes (*15*) are also suggested to have a similar structure.

Though it was not undertaken to compare the electrophoretic patterns with sedimentation patterns, it appears from the present study that the structure of the microsomal RNP particles is quite different from that of the viral nucleoprotein, and considerable part of RNA exist on the surface of the particles, because partial removel of RNA from the particles by autodegradation or with pancreatic RNase always resulted in the gradual decrease in their mobilities (component 2 → component 2) and therefore their electrophoretic mobilities seem to depend upon the RNA content of the particles.

If it is allowed to assume that the RNA of the RNP particles was composed of n subunits of RNA as suggested by Roth (*16*), and if we represent them as (n) RNA, then the degradation process of the particles may be expressed as follows;

1) (n)RNA-protein → (n − 1) RNA-protein + RNA →
(n − 2) RNA-protein + 2 RNA → .... (n − m) RNA-protein + (m) RNA
2) RNA → oligo-or mono-nucleotides.

The component 3 may be represented by (n − m) RNA-protein, though value of m may be different under what conditions and to what degrees the degradation was carried out.

The easiness with which RNA of the particles is degraded with RNase (*4*, *5*, *7*) and the remarkable turbidity increase of the RNP solution when incubated with RNase (*7*) also support the assumption that considerable RNA should exist on the surface of the particles. The physicochemical stability of the RNP particles in solution probably depends upon the negative charge of RNA on the surface of the particles, and this RNA seems to be playing some part in protein synthesis.

SUMMARY

The microsomal ribonucleoprotein (RNP) isolated by deoxycholate treatment was observed by Tiselius' electrophoretic method.

1. As it has been reported by Petermann that the dialysate of rat liver cytoplasm has a stabilizing effect on these particles, the microsomal supernatant was dialysed against the Miller-Golder's buffer, pH 7.0 and ionic strength 0.1, and the RNP particles were prepared in this "buffered dialysate". Electrophoretic analysis showed that the standard RNP solution prepared in such a manner had a large faster boundary (component 2) accompanied by a small component with somewhat slower mobility (component 3).

2. When the RNP was prepared in the Miller-Golder's buffer, pH 7.0 and ionic strength 0.1, the component 2 was considerable degraded with compensatory increase of the component 3. The mobility of the component 3 was also smaller than in the former case, and the appearance of a small free RNA boundary was occasionally noted.

3. Such degradations were accerelated either by the incubation of the RNP at 37° without adding enzyme or by the addition of pancreatic RNase to the RNP solution in the cold.

4. Incubation with trypsin at 37° destructed the RNP into two or three smaller components.

5. From these results, it was estimated that the mobility of the RNP particles depended at least partially on the RNA content of the particles and that considerable part of RNA should exist on the surface of the particles.

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The Journal of Biochemistry, Vol. 46, No. 9, 1959

CHROMATOGRAPHIC STUDIES ON GAMMA RAY-IRRADIATED ALBUMIN*

There are numerous factors which control the radiosensibility of protein solution, such as protein concentration, pH, salts, temperature, oxygen tension, dosage rate *etc.* (*1–4*). The effects of radiation on enzyme solutions have been studied by many investigators. Hardly any studies using subfractionation technique have been made on the effect of ionizing radiation on protein solution except by Rosen (*5*).

The authors have studied the effects of above stated factors on γ-ray irradiated Armours' bovine plasma albumin solution by using column chromatography, visicosity, ultraviolet absorption spectrum and amperometric titration of SH-radical (*6*, *7*). Chromatography was performed at 8～25° by using very coarse grain of hydroxylapatite (1.5×12 cm.) as an adsorbent, and 0.02 *M*, 0.04 *M*, 0.07 *M*, 0.11 *M* and 0.40 *M* Na-phosphate buffer (pH 6.8) as eluants (*8*, *9*). The elution rate was about 30～35 ml. per hour and the effluent was fractionated into fractions of 3.5 ml. each by using a fraction collector.

The protein content was analysed by the extinction at 280 mμ, because the extinction of unirradiated protein at 280 mμ was nearly equal to that of irradiated one in the authors' experiments.

From comparison of change of chromatogram with those of visicosity, ultraviolet absorption spectrum and SH-radical, it was found that the chromatogram was more sensitive towards radiation than other measurements. However, hydroxylapatite was poisoned by γ-ray-irradiated protein solution. The deactivated adsorbent was easily reactivated by heating with 0.01 *M* Na-phosphate buffer pH 6.8 (*9*).

20 mg. of bovine plasma albumin was dissolved in 60 ml. of redistilled water. The irradiation of γ-ray (Co^{60}) was performed at 20° in an open pyrex glass tube under the following conditions: dose rate 4×10^3 r. per hour, dose, $4/3 \times 10^3$ r., $8/3 \times 10^3$ r., 4×10^3 r., $16/3 \times 10^3$ r., $20/3 \times 10^3$ r. The irradiated solution was immersed in ice for 24 hours and chromatographed. One of the results is shown in Fig. 1. Only log (percentage of the 1st subfraction) was linear against dose of γ-ray. When the concentration was decreased, there was a proportional decrease in 37 per cent dose of the 1st subfraction (D_{37}). That is, D_{37} of 0.019 per cent, 0.038 per cent and 0.075 per cent were 3×10^3 r., 5.1×10^3 r., 7.5×10^3 r., respectively, at 16°, γ-ray source having an intensity of 4×10^3 r. per hour. Radiosensibility of albumin, also, was affected by γ-ray intensity as reported by Forsberg (*3*). D_{37} of 0.019 per cent, 0.038

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per cent and 0.075 per cent were 1.4×10^4 r., 1.6×10^4 r., and 2.2×10^4 r., respectively, at 26°, γ-ray having an intensity of 2.4×10^5 r. per hour.

Most of the investigations on radiosensibility of serum albumin, egg albumin etc. were directed to detect change of visicosity or ultraviolet absorption spectrum, which is most destructive process, occurring only after receiving a large dose of irradiation (*2*). On the other hand, the contradictory finding was obtained by using subfractionation technique. When the concentration of albumin and dose rate of γ-ray were decreased, 37 per cent dose of 1st subfraction will be as low as SH-enzyme.

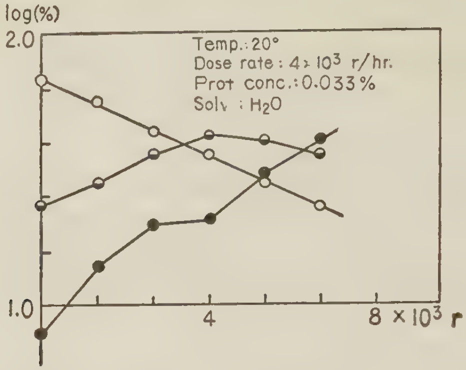

FIG. 1. Effect of γ-ray on chromatogram of bovine plasma albumin. ○, ◒ and ● showed three subfractions which were eluted with 0.07 *M*, 0.11 *M* and 0.40 *M* Na-phosphate buffer (pH 6.8), respectively. The ordinate and the abscissa showed log per cent of three subfractions and dose of γ-ray, respectively. See text for other experimental conditions.

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ACTIVATION OF MYOSIN A ADENOSINETRIPHOSPHATASE BY SOME ORGANIC SOLVENTS*

A number of substances of different kinds have been known to have a remarkable activating action on myosin ATPase, for example, PCMB (Kielley and Bradley (*1*) 1956) and PMA (Greville and Needham (*2*) 1955; Perry and Chappell (*3*) 1954, 1957), EDTA (Bowen and Kerwin (*4*) 1954; Friess (*5*) 1954) and its analogues (Bowen and Kerwin (*4*) 1954; Ebashi, Ebashi and Fujie (*6*) 1959), and DNP (Greville and Needham (*2*) 1955; Perry and Chappell (*3*) 1954, 1957). It is worth noting that some of these compounds also have a relaxing action on glycerinated muscle fibers (Watanabe (*7*) 1955, 1957; Bozler (*8*) 1954; Watanabe (*9*) 1957; Ebashi, Ebashi and Fujie (*6*) 1959) in the presence of ATP, although their relaxing actions are not in parallel with their accelerating effects on myosin ATPase (Ebashi, Ebashi and Fujie (*6*) 1959).

Recently acetone and some other organic solvents (Ebashi and Ebashi (*10*, *11*) 1959) have been shown as a new relaxing agent on glycerinated muscle fibers. In view of the facts mentioned above, it is interesting to know the effects of these compounds on myosin ATPase.

As shown in Fig. 1, *n*-butyl alcohol and isobutyl alcohol exerted a remarkable acceleration, more than five times, on myosin A ATPase in the presence of 0.6 M KCl at pH 8.0. All other compounds so far tested also showed more or less activation. In the series of alcohols, the order of their activating activities was the same as that of their relaxing activities measured by isometric method (Ebashi and Ebashi (*11*) 1959), *i.e.*, *n*-butyl alcohol > isobutyl alcohol > *n*-propyl alcohol > ethyl alcohol. This may be, however, an accidental coincidence, because ethyl acetate, the strongest relaxing agent among the organic solvents so far, (Ebashi and Ebashi (*10*, *11*) 1959) did not show so marked acceleration on myosin ATPase as produced by butyl alcohols.

The accelerating effect on myosin ATPase was very sensitive to Mg^{++}; even $1\times10^{-4}\,M$ of Mg^{++} almost abolished the activating effect.

The mechanism of the activation of myosin ATPase by these organic solvents is still unknown as in other cases. Further studies are now in progress and will be published later.

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* The following abbreviations are used in this letter: ATP, adenosinetriphosphate; ATPase, adenosinetriphosphatase; PCMB, *p*-chloromercuribenzoate; PMA, phenylmercuric acetate; EDTA, ethylenediamine tetraacetate; DNP, 2:4 dinitrophenol; Tris, tris-(hydroxymethyl)-aminomethane.

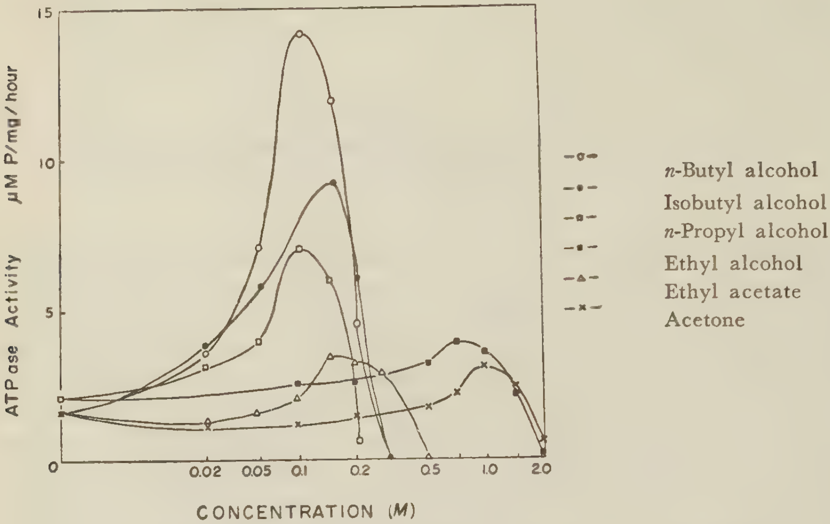

FIG. 1. Effects of some organic solvents on the ATAasc activity of myosin A.

Abscissa: the molar concentration of each compound. Ordinate: μM of inorganic phosphate split from ATP per mg. of myosin A per hour. Each millilitar of reaction mixture contained 1 μM of ATP, 600 μM of KCl, 20 μM of Tris (pH 8.0), about 0.1 mg. of myosin A, prepared by the method of Szent-Györgyi (*12*), and a specified amount of a compound to be tested. Incubations were conducted at 25° for 15 minutes.

We wish to express our thanks to Prof. H. Kumagai for his encouragement.

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LETTERS TO THE EDITORS

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EFFECT OF PRE-ILLUMINATION ON THE FORMATION OF PHYCOBILIN PIGMENTS IN A BLUE-GREEN ALGA, TOLYPOTHRIX TENUIS

It has been reported in a previous paper (*1*) that a certain light process (or processes) is involved in the formation of phycobilin pigments in a blue-green alga, *Tolypothrix tenuis*, and that the formation of each pigment species (phycocyanin, phycoerythrin and allophycocyanin) was provoked by the light of different spectral ranges. In the present study, it is shown that the formation of these pigments can be evoked by pre-illumination followed by dark incubation of algal cells in the presence of a nitrogen source.

Cells of *Tolypothrix tenuis** were suspended in a nitrogen-free medium** (4 to 10 ml. packed cells per liter) contained in an oblong flat flask and placed in a water thermostat regulated at 22 – 24° and illuminated from outside with a fluorescent light (20,000 Lux), or with a colored light which was obtained by using a red filter (Filter R-1 ; see previous paper (*1*)) to eliminate the lights shorter than 600 mμ. The algal suspension was continuously aerated with air containing 5 per cent CO_2. After about 24 hours of incubation in the light, the algal cells collected by centrifugation were resuspended in a medium containing KNO_3 (3.0 g. per liter) together with other essential mineral nutrients (20 to 40 ml. packed cells per liter) and shaken at 32° in the dark. Determination of the pigment content was performed with aliquots of the suspension which were taken out at appropriate time intervals. The samples were immediately frozen with NaCl-ice mixture, to stop the further progress of the reactions and stored in the frozen state. The contents of phycoerythrin and phycocyanin were estimated by the method described in a previous paper (*3*) and chlorophyll and carotenoids by Mackinney's method (*4*). No attempt was made to estimate allophycocyanin content because of the technical difficulties in obtaining accurate values.

During the period of pre-illumination, there occurred a distinct decrease in the levels of phycobilins (Fig. 1), while the levels of chlorophyll and carotenoids remained practically unchanged. This may be due to the bleaching action of the strong light applied (*2*), to which phycobilin pigments are more vulnerable than chlorophylls and carotenoids.

The cells which had been pre-illuminated with unfiltered fluorescent light readily formed phycoerythrin in the subsequent dark incubation, provided that nitrate was supplied at an appropriate stage in the dark incubation (Fig. 1-A).

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* Fine pieces of the algal tissues which had been cultured for about 2 weeks by the method described in a previous paper (*2*).

** Composition : (per liter) $K SO_4$, 2.6 g. ; $MgSO_4 \cdot 7H_2O$, 0.5 g. ; $Na_2HPO_4 \cdot 12H_2O$, 0.5 g. ; $CaCl_2$, 0.02 g. ; $FeSO_4$, 0.004 g. ; Arnon's A_5 solution, 1 ml.

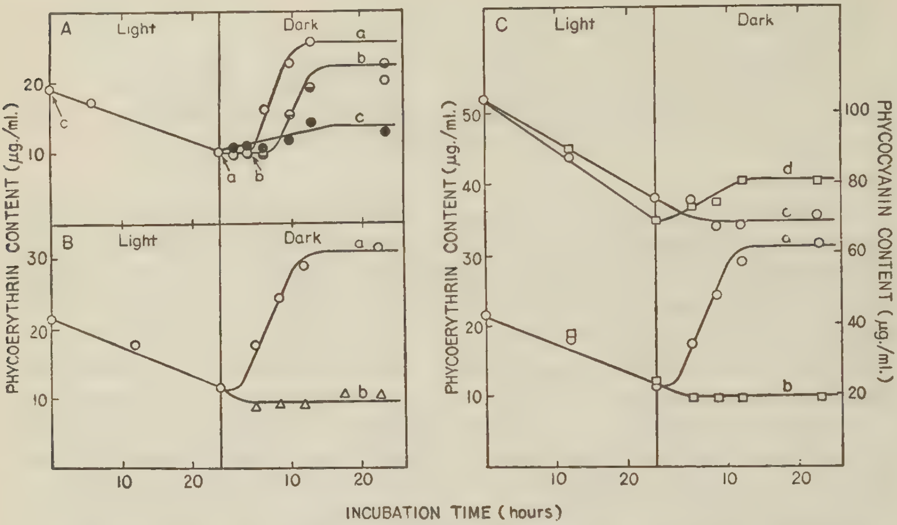

FIG. 1. Time course of phycobilin formation in pre-illuminated *Tolypothrix* cells.

A. Effect of nitrate on phycoeythrin formation in cells which had been pre-illuminated with unfiltered fluorescent light; Curve a: nitrate added at zero time of dark incubation, Curve b: nitrate added after 6 hours, Curve c: nitrate added at the beginning of pre-illumination. The arrows indicate the time of addition of nitrate.

B. Effect of anaerobiosis on phycoerythrin formation in cells which had been pre-illuminated with unfiltered fluorescent light; Curve a: aerobic (gas space: air), Curve b: anaerobic (gas space: N_2).

C. Formation of phycoerythrin and phycocyanin as induced by pre-illumination with filtered (red) and unfiltered fluorescent light; Curve a and c: pre-illumination with unfiltered light, Curve b and d: pre-illmination with filtered light (red). Experimental conditions; see text.

If nitrate was added within the first 6 hours of the dark period, the formation of phycoerythrin took place after an induction period of about 4 hours. When nitrate was added more than 10 hours after the beginning of the dark incubation, there occurred no positive formation of the pigment. The presence of nitrate during the pre-illumination period seems to be rather unfavorable for the production of the pigment. No pigment formation occurred under anaerobic conditions (Fig. 1-B). The final level of phycoerythrin, which was attained after about 14 hours of dark incubation, varied with the time of application of nitrate (Fig. 1-A). It should be remarked that under the conditions mentioned above, no increase or even a slight decrease in the phycocyanin content was observed (Fig. 1-C).

When, however, the algal cells were pre-illuminated with red light, the formation of phycocyanin did occur, while no formation of phycoerythrin took place (Fig. 1-C). Here also the presence of nitrate was found to be an indispensable condition for the pigment formation. It should be added that no significant changes in the levels of chlorophyll and carotenoids were observed under the condition of phycobilin formation mentioned above.

The data presented above indicate that the photochemical formations of phycobilin pigments are the processes involving certain dark reactions preceded by photochemical reactions. Conceivably, some precursors of the pigments are accumulated in the light and converted in the dark into the pigments. Worthy of note is the fact that the spectral ranges of the lights which proved to be effective in causing the subsequent formation in the dark of each phycobilin pigments were the same as those of the lights which were found to be effective in the experiments of continuous illumination performed in the previous study (*1*) (the lights of around 500 mμ being effective for phycoerythrin formation and those of 550–700 mμ effective for phycocyanin formation).

The fact that, in the pre-illumination experiments, the pigment formation in the dark could occur only in the presence of a nitrogen source and under aerobic conditions indicates that the reaction occurring in the dark are rather intricate synthetic processes involving nitrogen and entailing expenditures of respiratory energies.

In both the precedent and present experiments, it was observed that a condition which accelerated the formation of one phycobilin pigment depressed the formation of the other. This fact makes it probable to assume that the two phycobilin pigments are formed from one and the same precursor. More detailed investigations concerning the mechanism of phycobilin formation are now in progress.

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